UNIVERSIDADE FEDERAL DE MINAS GERAIS
Instituto de Ciências Biológicas
Programa de Pós Graduação em Bioquímica e Imunologia

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POTENCIAL TERAPÊUTICO DO FUNGO *AGARICUS BLAZEI* NO TRATAMENTO DE DIFERENTES FORMAS CLÍNICAS DE LEISHMANIOSES E ESTUDO DO ENVOLVIMENTO DA PROTEÍNA NLRP12 NA INFECÇÃO POR *Leishmania chagasi*

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Tese apresentada como requisito parcial para obtenção do grau de Doutor em Imunologia, pelo Departamento de Bioquímica e Imunologia do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais.

Orientador: Carlos Alberto Pereira Tavares

Coorientador: Eduardo Antonio Ferraz Coelho

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ATA DA DEFESA DA TESE DE DOUTORADO DE DIOGO GARCIA VALADARES. Aos sete dias do mês de novembro de 2013 às 14:00 horas, reuniu-se no Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, a Comissão Examinadora da tese de Doutorado, indicada *ad referendum* do Colegiado do Curso, para julgar, em exame final, o trabalho intitulado "Potencial terapêutico do fungo *Agaricus blazei* no tratamento de diferentes formas clínicas das *Leishmanioses*", requisito final para a obtenção do grau de Doutor em Ciências: Imunologia. Abrindo a sessão, o Presidente da Comissão, Profa. Carlos Alberto Pereira Tavares, da Universidade Federal de Minas Gerais, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra ao candidato para apresentação de seu trabalho. Seguiu-se a arguição pelos examinadores, com a respectiva defesa do candidato. Logo após a Comissão se reuniu, sem a presença do candidato e do público, para julgamento e expedição do resultado final. Foram atribuídas as seguintes indicações: Dra. Santuza Maria Ribeiro Teixeira (Universidade Federal de Minas Gerais), aprovado; Dr. André Gustavo Tempone Cardoso (Instituto Adolfo Lutz/SP), aprovado; Dr. Alexandre Barbosa Reis (Universidade Federal de Ouro Preto), aprovado; Dr. Gustavo Batista de Menezes (Universidade Federal de Minas Gerais), aprovado; Dr. Eduardo Antonio Ferraz Coelho-co-orientador (Universidade Federal de Minas Gerais), aprovado; Dr. Carlos Alberto Pereira Tavares - Orientador (Universidade Federal de Minas Gerais), aprovado. Pelas indicações o candidato foi considerado APROVADO. O resultado final foi comunicado publicamente ao candidato pelo Presidente da Comissão. Nada mais havendo a tratar, o Presidente da Comissão encerrou a reunião e lavrou a presente Ata que será assinada por todos os membros participantes da Comissão Examinadora. Belo Horizonte, 07 de novembro de 2013.

Dra. Santuza Maria Ribeiro Teixeira (UFMG)

Dr. André Gustavo Tempone Cardoso (Instituto Adolfo Lutz/SP)

Dr. Alexandre Barbosa Reis (Universidade Federal de Ouro Preto)

Dr. Gustavo Batista de Menezes (UFMG)

Dr. Eduardo Antonio Ferraz Coelho-co-orientador (UFMG)

Dr. Carlos Alberto Pereira Tavares - Orientador (UFMG)

Prof. Jader dos Santos Cruz
Coordenador de Curso de Pós Graduação
em Bioquímica e Imunologia
ICB - UFMG

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“Inventar é imaginar o que ninguém pensou; é acreditar no que ninguém jurou; é arriscar o que ninguém ousou; é realizar o que ninguém tentou. Inventar é transcender."

(Santos Dumont)

RESUMO

As leishmanioses são doenças negligenciadas que podem causar morbidade e mortalidade nos pacientes acometidos. Apesar de existirem algumas alternativas de tratamento, os fármacos utilizados podem causar toxicidade elevada nos indivíduos, o que dificulta a adesão dos mesmos ao tratamento e pode prejudicar a eficácia dos produtos. Buscando minimizar esses problemas referentes ao tratamento disponível para as diferentes formas clínicas das leishmanioses, no presente trabalho, um extrato aquoso do cogumelo do sol *Agaricus blazei* foi preparado e a atividade leishmanicida do mesmo foi avaliada contra diferentes espécies de *Leishmania*, a saber, *Leishmania amazonensis*, *L. major* e *L. chagasi*. O extrato mostrou-se eficaz na redução da viabilidade celular dos parasitas e o tratamento de macrófagos infectados utilizando o extrato mostrou uma redução significativa da carga de parasitas intra-macrófagos. O tratamento prévio de formas promastigotas com o extrato mostrou uma redução expressiva da capacidade infectiva dos mesmos, revelando um potencial preventivo do extrato do fungo contra a infecção por *Leishmania*. Outro fator importante foi a ausência de citotoxicidade demonstrada pela preparação, tanto em macrófagos peritoneais murinos quanto em eritrócitos humanos. A eficácia terapêutica do extrato foi avaliada em camundongos BALB/c infectados com *L. amazonensis* e o tratamento oral se mostrou eficaz na redução do inchaço no local da infecção e na redução da parasitemia em diferentes tecidos e órgãos avaliados (lesão da pata, baço e linfonodo). Esplenócitos dos animais tratados com o extrato apresentaram níveis elevados de IFN-gama e óxido nítrico nas culturas estimuladas *ex vivo*, e a preparação terapêutica não apresentou toxicidade hepática nos animais tratados. Com o objetivo de se conhecer um pouco mais o extrato foi feito o fracionamento e a análise fitoquímica, e cinco frações semi-purificadas foram obtidas. Tais produtos foram avaliados quanto a sua atividade leishmanicida e a fração F.a.b 5 foi selecionada como a apresentando o melhor resultado. Em seguida foi avaliada a eficácia terapêutica do extrato aquoso e F.a.b 5 em modelo visceral com a infecção por *L. chagasi*, em diferentes regimes de tratamento, inclusive como um esquema químio-profilático. Os diferentes tipos de tratamento mostraram eficácia em reduzir a carga parasitária no fígado, baço e linfonodo dos animais, sendo que no baço e linfonodo a redução foi maior que o do tratamento de referência utilizado como controle, a anfotericina B. A quimio-profilaxia utilizando F.a.b 5 foi aquela que apresentou melhor eficácia terapêutica, com os menores níveis de carga parasitária observada no baço e linfonodo dos animais. Assim como no modelo de leishmaniose cutânea, os animais infectados com *L. chagasi* e tratados pelo

extrato e Fa.b 5 mostraram uma maior resposta Th1, evidenciada pelos níveis elevados de IFN-gama e pela redução dos níveis de IL-4 nas culturas esplênicas estimuladas *ex vivo*. Dessa forma, a utilização do fungo *A. blazei* mostrou-se eficaz seja no tratamento convencional ou em uma forma de quimio-profilaxia tanto nos modelos de leishmaniose tegumentar quanto visceral murinas avaliadas.

Com o propósito de aperfeiçoar as técnicas de avaliação de eficácia terapêutica pré-clínica pela aquisição do conhecimento do uso de imageamento *in vivo*, um projeto em conjunto com a pesquisadora Dra Mary E. Wilson (University of Iowa, USA) foi desenvolvido para avaliar o papel da proteína NLRP12 na infecção por *L. chagasi*. Nesse estudo, além de verificar a aplicabilidade do imageamento *in vivo* para os modelos pré-clínicos de leishmanioses, foi observado um papel de controle da inflamação desempenhado pela NLRP12, sendo observado um aumento da infiltração de leucócitos no sítio da inflamação, como neutrófilos e monócitos inflamatórios nos animais deficientes em NLRP12. Além disso, pode-se comprovar o papel de NLRP12 na migração de células dendríticas, porque tais células se tornam não-responsivas na ausência dessa proteína causando mudanças na resposta adaptativa devido à redução da migração dessas células no baço e uma menor apresentação de antígenos.

Palavras-chave: *Agaricus blazei*, leishmanioses, tratamento, atividade leishmanicida, proteína NLRP12, imageamento, inflamação.

ABSTRACT

Leishmaniasis is a neglected disease that causes morbidity and mortality in affected patients. Although there are few treatment alternatives, the drugs present toxicity to individuals, reducing the adhesion to treatment which may impair the effectiveness of the products. Seeking to minimize these problems that concern the treatments available for the different clinical forms of leishmaniasis, in the present thesis, an aqueous extract of the *Agaricus blazei* Murril was prepared and it's leishmanicidal activity was evaluated against different *Leishmania* species, namely *Leishmania amazonensis*, *L. major* and *L. chagasi*. The extract was effective in reducing the cellular viability of parasites and experiments treating infected macrophages with the extract showed a significant reduction of the intra-macrophages parasite load. Pretreatment of promastigotes with the extract showed a significant reduction of its infective capacity, showing a preventive potential of the fungus extract against *Leishmania* infection. Another important factor was the absence of cytotoxicity demonstrated by the preparation, both in murine peritoneal macrophages as in human erythrocytes. The therapeutic efficacy of the extract was evaluated in BALB/c mice infected with *L. amazonensis* by oral treatment, and it was effective in reducing the swelling at the site of infection as well as in the reduction of parasitemia in evaluated organs (paw, spleen and lymph node). Splenocytes from animals treated with the extract showed elevated levels of IFN-gamma and nitric oxide in cultures stimulated *ex vivo*, and the preparation showed no liver toxicity in the treated animals. Then the fractionation and phytochemical analysis of the extract and five semi-purified fractions were performed. Such products were evaluated for their antileishmanial activity and the fraction Fab 5 was selected as presenting the best results. The therapeutic effectiveness in a model for visceral infection with *L. chagasi* was also evaluated using aqueous extract or Fab 5 in different treatment regimens, including a chemo-prophylactic regimen. The different types of treatment were effective in reducing the parasitic load in the liver, spleen and lymph node of the animals. The reduction observed in the lymph node and spleen was greater than in the animals treated with the drug used as control, amphotericin B. The chemoprophylaxis using Fab 5 showed better therapeutic efficacy with lower levels of parasite load observed in the spleen and lymph node of the animals. As in the model of cutaneous leishmaniasis, animals infected with *L. chagasi* and treated with extract and Fa.b 5 showed a higher Th1 response, marked by high IFN-gamma levels and reduced levels of IL-4 in *ex vivo* stimulated spleen

cultures. Thus, the use of *A. blazei* fungus, in different treatment strategies (conventional treatment as well chemoprophylaxis) was effective in the treatment of both murine cutaneous and visceral models of leishmaniasis. With the purpose of improving the techniques for evaluating the preclinical therapeutic efficacy of drugs in leishmaniasis models, through the acquisition of knowledge in *in vivo* imaging technique, a project in conjunction with Dr. Mary E. Wilson (University of Iowa, USA). The objective of the project was to evaluate the role of NLRP12 protein in the infection caused by *L.i chagasi*. In this study, besides verifying the applicability for *in vivo* imaging in preclinical models of leishmaniasis, we could detect a controlling role in inflammation played by NLRP12, in which could be observed an increase in leukocyte infiltration at the site of inflammation such as neutrophils and inflammatory monocytes in the NLRP12 deficient mice. Furthermore, it could be proved the role of NLRP12 in the dendritic cells migration and also that these cells become non-responsive against chemokines in the absence of this protein. This reduced migration causes changes in the adaptive response due to a reduced antigen presentation in the spleen.

Key-words: *Agaricus blazei*, leishmaniosis, treatment, leishmanicidal activity, NLRP12 protein, *in vivo* imaging, inflammation.

LISTA DE SIGLAS E ABREVIATURAS

AbM	*Agaricus blazei* Murril
AHCC	Active Hexose Correlated Compound
AIM 2	Absent in melanoma 2
ALT	Alanine transaminase
APAF-1	Apoptotic protease activating factor 1
AmpB	Anfotericina B
ASC	Apoptosis-associated speck-like protein containing a CARD
AST	Aspartate aminotransferase
ATL	American tegumentar leishmaniasis
ATOM	Antitumor organic substance Mie
CARD	Caspase activation and recruitment domain
CC50	Citotoxity concentration 50%
CCL	Chemokine (C-C motif) ligand
CL	Cutaneous leishmaniasis
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-X-C motif) receptor
DAMP	Damage-associated molecular pattern
DCL	Diffuse cutaneous leishmaniasis
dLN	Draining lymph node
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic acid
Fab	Fraction of *Agaricus blazei*
FBS	Fetal Bovine Serum
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBSS	Hank's Balanced Salt Solution
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HPLC	High-performance liquid chromatography
IC50	Inhibitory concentration 50%
IL	Interleukine

INF-Y	Interferon gamma
iNOs	Inducible nitric oxide synthase
IVIS	In vivo imaging system
LCM	*L.i.c* promastigote meidum culture
L.i.c	*Leishmania infantum chagasi*
LCD	Leishmaniose cutânea difusa
LPS	Lipopolysaccharide
LRR	Leucine-rich repea
LT	Leishmaniose tegumentar
Luc	Luciferase
LV	Leishmaniose visceral
M-CSF	Macrophage colony-stimulating factor
MCL	Mucocutaneous leishmaniasis
MHC	Major histocompatibility complex
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole
MyD88	Myeloid differentiation primary response gene (88)
NACHT	[NAIP (neuronal apoptosis inhibitory protein), CIITA (MHC class II transcription activator), HET-E (incompatibility locus protein from *Podospora anserina*) and TP1 (telomerase-associated protein)]
NBS	Nucleotide binding site
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	Nucleotide-binding oligomerization domain receptors
NOD	Nucleotide-binding oligomerization domain-containing protein
NOS 2	Nitric oxide synthase
PAMP	Pathogen-associated molecular patterns
PFA	Paraformaldeído
PMN	Polymorphonuclear leukocytes
PRR	Pattern recognition receptors

PYD	Pyrin domain
PYHIN	Pyrin and HIN domain-containing protein family
RBC50	Red blood concentration 50%
RIG-1	Retinoic acid-inducible gene 1
RPMI	Roswell Park Memorial Institute
RPMI-PR-	Roswell Park Memorial Institute phenol red negative
SLA	Soluble Leishmania antigen
TNF-α	Tumor necrosis factor α
VL	Visceral leishmaniasis

SUMÁRIO

INTRODUÇÃO

As leishmanioses são doenças tropicais que apresentam incidência em cerca de 98 países, com 350 milhões de pessoas expostas aos riscos de infecção e 12 milhões de pessoas clinicamente afetadas (WHO 2010).

As opções de tratamento disponíveis para a leishmaniose visceral (LV) possuem problemas relacionados com a eficácia, com os efeitos adversos e custos elevados, tornando o tratamento uma questão complexa. A ocorrência de resistência dos parasitas aos fármacos comumente utilizados no tratamento das leishmanioses, bem como a elevada toxicidade que os mesmos podem causar nos pacientes tratados, tem causado preocupação nas autoridades de Saúde Pública. Fármacos de segunda linha, tais como a anfotericina B, paromomicina e miltefosina são alternativas, mas se limitam a cumprir os requisitos de uma droga segura (Moore & Lockwood 2010). Tais fatos evidenciam a necessidade de se buscar novas opções para o tratamento efetivo das leishmanioses.

Apesar do Brasil possuir uma das maiores biodiversidades do planeta, pouco ainda é explorado na produção de fármacos oriundos de organismos encontrados em território nacional. Grande parte dos estudos realizados nas triagens de substâncias ativas contra *Leishmania* é realizada com espécies vegetais, sendo que poucos são realizados com substancias retiradas de animais e mais raros ainda são estudos realizados com fungos (Rupashree Sen & Mitali Chatterjee 2011).

O fungo *Agaricus blazei* é um cogumelo nativo do Brasil e comercializado por algumas empresas, inclusive a Minasfungi do Brasil. Alguns trabalhos existentes na literatura revelaram a atividade antimicrobiana do *Agaricus blazei* (Sakagami et al., 1991), mas nenhum explorou seu potencial para uso no tratamento das leishmaioses. Dessa forma, este trabalho teve como objetivo geral avaliar o potencial de *Agaricus blazei* para ser utilizado como uma forma de tratamento alternativo em diferentes formas clínicas de leishmanioses.

A tese é apresentada em capítulos sendo cada um correspondente a um trabalho científico publicado ou submetido a publicação. Para melhor compreensão dos problemas abordados foi feita uma revisão da literatura para apresentar as propriedades nutracêuticas do *cogumelo Agaricus blazei* e a fundamentação teórica sobre a necessidade de melhoria no tratamento das leishmanioses.

Parte 1
REVISÃO DA LITERATURA

1. Cogumelo-do-sol *Agaricus blazei*

Agaricus blazei é um fungo aeróbio que tem o potencial de degradar matéria orgânica rica em celulose, hemicelulose e lignina para a obtenção de energia. Este cogumelo foi reclassificado por Wasser (2002) para *Agaricus brasilienses*, porém, a denominação de *Agaricus blazei* tem sido a mais usada na literatura científica (Amazonas, 2004) (Figura 1). O *A. blazei* vem sendo utilizado como nutracêutico, por possuir elevado potencial na área da indústria alimentícia e na farmacêutica como medicamento. Registre-se que o termo nutracêutico se refere aos alimentos funcionais que, consumidos como parte da dieta cotidiana, possuem valores nutricionais elevados e beneficiam a saúde (Miles & Chang, 1997; Wasser & Weis, 1999). No caso em comento, o fungo é consumido por seu elevado valor protéico, mineral e vitamínico.

Figura 1: Cogumelo *Agaricus blazei*. Fonte: Minasfungi do Brasil Ltda.

O fungo é utilizado em função de seus efeitos no bem-estar geral da saúde das pessoas. As principais atividades descritas na literatura são o controle da diabetes do tipo II (Hsu et al.

2007), da hipertensão arterial e osteoporose (Bellin et. al. 2003), na recaptação de cálcio por meio do ergosterol (Kasai et. al. 2004) e no tratamento do câncer e AIDS, sendo que foi comprovado que o uso do fungo fortalece o sistema de defesa natural dos pacientes imunossuprimidos (Gao et. al. 2007). Estas propriedades estão relacionadas às substâncias presentes no *A. blazei*, tais como o β-D-glucans, cerebrosídeos, esteróides, ergosterol e ácidos graxos (Mizuno, 2002).

Desde o início da década de 90, *A. blazei* é cultivado no Brasil em escala comercial, cuja produção tem como principal destino o mercado de exportação, sendo o Japão o maior comprador (Braga et a. 1998). *A. blazei* é comercializado nas formas desidratado em pó ou fatiado, de chá (extrato aquoso quente) ou na forma de suco (extração aquosa a frio), a partir da infusão dos cogumelos secos em água (Eira et al., 1996).

Esse cogumelo possui grande valor nutricional por possuir aminoácidos essenciais, vitaminas e altos teores de fibras, possuindo também outras substâncias de potencial valor medicamentoso, como β-D-glucans, glucina, ergosterol, adrenocromo, *composto de hemicelulose ativa* (AHCC), *substância orgânica antitumor mie* (ATOM), pró-vitamina C e alcalóides. Os cogumelos são alimentos com poucas calorias e que apresentam de 85% a 90% de água. Quando desidratados, são ricos em proteínas e carboidratos, contendo de 40% a 45% de proteína bruta, 38% a 45% de carboidratos, 6% a 8% de fibra, 5% a 7% de cinzas e 3% a 4% de lipídeos, com predominância do ácido linoléico (70% a 78%). Contêm vitaminas do complexo B (B1 e B2), niacina e grande quantidade de potássio (3%), além de outros minerais, como P, Mg, Ca, Na, Cu, Zn, Fe, Mn e Mo (Urben 2004).

Em países como Japão, Rússia, China e Estados Unidos diferentes polissacarídeos com atividades antitumorais foram extraídos do corpo de frutificação e micélio de várias espécies de cogumelos medicinais. A ação imunomoduladora dos cogumelos é devida, principalmente, a ativação de macrófagos (Ito et al. 1997, Wasser & Weis, 1999), a estimulação de células natural killer (NK), e há evidências do aumento da produção de substâncias pró-inflamatórias (Kaneno et al., 2004, Bernardshaw et al. 2005; Kawamura & Kasai, 2006; Yuminamochi et al., 2007). Sorimachi et al. (2001) observaram que componentes de *A. blazei* são capazes de ativar macrófagos, resultando em um aumento na produção de citocinas tais como TNF-α, IL-8 e NO.

Além das propriedades imunoestimuladoras, esses cogumelos causam uma redução significativa nos níveis de colesterol no sangue, reduzem a hiperlipidemia e exibem ação antitrombótica (Bobek et al, 1991 a, b; Gunde-Cimerman et al, 1993, 1995; Mizuno, 1995). O principal mecanismo de ação do fungo *Agaricus blazei* aparenta estar relacionado com a atividade biológica de polissacarídeos, principalmente, o chamado (1→6)-(1→3)-β-D-glucan (Figura 2), encontrado nas frutificações. A fração solúvel em água, avaliada sobre o Sarcoma 180 em ratos, apresentou 97% de ação inibitória no crescimento de tumores sólidos, além de um aumento significativo na proliferação de linfócitos T e B *in vitro* (Dong et al., 2002).

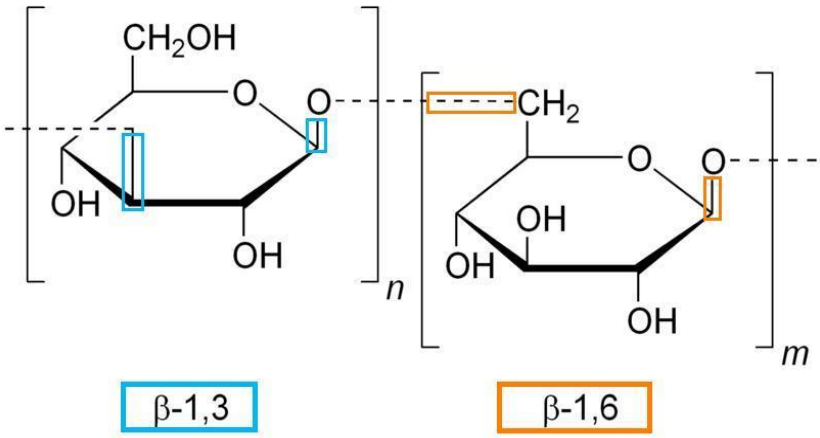

Figura 2: Estrutura química dos β-D-glucanos. Fonte: Ooi VE, Liu F (2000).

Os β-D-glucans de *A. blazei* apresentaram toxicidade específica sobre células tumorais, contudo, não apresentaram toxicidade frente às células normais (Fujimiya et al. 1998; Shimizu et al., 2002). Takaku et al. (2001) isolaram ergosterol de *A. blazei* e mostraram que o mesmo apresentava atividade antitumoral. O ergosterol foi também encontrado em *Lentinula edodes* (*Shiitake*) e *Polyporus umbellatus*. Esses resultados sugerem que o ergosterol e seus metabólitos possam estar envolvidos na inibição da angiogênese (Leif et al. 2006, Zhou et al. 2007).

Ito et al. (1997) observaram que a inibição de diferentes tipos de tumores em ratos foi mediada pela ativação de macrófagos e alteração do componente C3 do complemento, por meio do complexo de polissacarídeos e proteínas "ATOM" (Antitumor Organic Substance Mie), extraído do *A. blazei*. Shimizu et al. (2002) demonstraram a inibição de células tumorais

através da ativação, dependente da via alternativa do complemento, pelo *A. blazei* e a formação de um complexo opsonizante entre *A. blazei* e C3bi no soro humano.

Em outro estudo, 20 pacientes com leucemia aguda tratados com quimioterapia foram distribuídos nos seguintes grupos: (a) grupo experimental (20 gramas de *Agaricus blazei*, 3 vezes ao dia; *n*=10) e (b) grupo controle (placebo; *n*=10). No grupo experimental, oito pacientes alcançaram remissão tumoral completa, dois pacientes permaneceram sem remissão e os valores de hemáceas e granulócitos no sangue periférico retornaram aos níveis normais em um período de 7 a 8 dias, ao final do tratamento. No grupo controle, cinco pacientes alcançaram remissão completa, dois pacientes tiveram remissão parcial e três não apresentaram remissão do tumor (Shimizu et al., 2002).

Segundo estudos realizados por Martins (2004), a administração de *A. blazei* na forma de extrato aquoso em cobaias foi capaz de inibir o crescimento do tumor de Ehlich. Estudos com animais transplantados com sarcoma 180 indicaram que o polissacarídeo de *A. blazei* induziu ao aumento do número de células envolvidas na imunidade. Houve uma diferença significativa na população de linfócitos e neutrófilos, em porcentagens no sangue periférico dos animais transplantados, quando comparados aos do grupo controle. Os resultados deste estudo sugerem que os polissacarídeos possuem efeitos antitumorais *in vivo*, fato que reforça o potencial anticancerígeno dos cogumelos e que tal atividade poderia estar relacionada às propriedades imunoestimulatórias do fungo (Gonzaga et al., 2009).

Além da sua atividade antitumoral, estudos têm demonstrado que polissacarídeos de cogumelos tem propriedades antimicrobianas (Sakagami et al., 1991), antivirais (Sorimachi et al., 1990), hepatoprotetoras (Ooi, 1996) e antifibrótica (Park et al., 1997), fatos que contribuíram para sua avaliação contra parasitas protozoários do gênero *Leishmania*.

2. Leishmanioses

As leishmanioses são doenças causadas por espécies de parasitas protozoários pertencentes ao gênero *Leishmania* que podem causar desde lesões cutâneas únicas de cura espontânea até a forma visceral, fatal, se não tratada (Desjeux, 2004). Dados da Organização Mundial de Saúde (WHO) indicam que há incidência da doença em 98 países no mundo, dos quais 72 são países em desenvolvimento. A incidência anual estimada é de 1.0 a 1.5 milhões

de novos casos de leishmaniose tegumentar (LT) e cerca de 500 mil casos de leishmaniose visceral (LV). Cerca de 90% dos casos de LT ocorrem no Brasil, Afeganistão, Argélia, Irã, Peru, Arábia Saudita e Síria; enquanto que os casos de LV concentram-se nas áreas rurais e suburbanas do Brasil, Bangladesh, Índia, Nepal e Sudão (Shaw, 2007).

Os parasitas pertencentes ao gênero *Leishmania spp.* são protozoários da ordem Kinetoplastida e da família Trypanosomatidae. Diversas espécies de *Leishmania* já foram descritas, podendo ser apontadas como causadoras de diferentes formas clínicas da doença. A classificação das espécies é feita em sub-gêneros: *Viannia* e *Leishmania*, de acordo com o desenvolvimento das mesmas no tubo digestivo do inseto vetor. Assim, as espécies do sub-gênero *Leishmania* desenvolvem-se na porção média e anterior do tubo digestivo do inseto vetor, enquanto as espécies do sub-gênero *Viannia* desenvolvem-se no intestino posterior do mesmo (Lainson et al, 1985).

Embora as leishmanioses sejam consideradas zoonoses, com transmissão silvestre ou em ambientes rurais, verificam-se, atualmente, alterações no padrão de transmissão devido às modificações sócio-ambientais, tais como desmatamento e o processo migratório que levam o homem do campo à periferia das grandes cidades (Gontijo & Melo, 2004). Dados epidemiológicos revelam a peri-urbanização e urbanização da LV no Brasil, devido à constatação de surtos da doença ocorridos em capitais como Rio de Janeiro, Belo Horizonte, Teresina, Natal, São Luiz e Fortaleza, em cidades como Araçatuba – SP, Santarém – PA, Corumbá – MS, Camaçari – BA e epidemias em Três Lagoas – MS, Campo Grande – MS e Palmas – TO (Cruz et al., 2009).

Outro aspecto que tem apresentado importância clínica e epidemiológica é a co-infecção entre o vírus HIV e *Leishmania*. As leishmanioses podem modificar a progressão da doença pelo HIV e a imunossupressão causada pelo vírus, de forma similar, podem facilitar a progressão da doença. A co-infecção é considerada doença emergente de alta gravidade em diversos países no mundo e calcula-se um crescimento contínuo devido à co-existência das duas infecções (Alvar et al., 2008).

2.1. Morfologia e ciclo biológico

O parasita *Leishmania* apresenta duas formas evolutivas principais: promastigota e amastigota. As formas promastigotas são formas alongadas, flageladas, móveis, com núcleo

único, cinetoplasto localizado entre a porção anterior e o núcleo e que se multiplicam no trato digestivo do inseto vetor. As formas amastigotas são formas arredondadas, com flagelo rudimentar, cinetoplasto em forma de bastão e que se multiplicam nas células do sistema fagocítico-monocitário do hospedeiro mamífero (Grimaldi & Tesh, 1993).

Diferentes espécies de mamíferos, dentre os quais roedores e canídeos, são reservatórios naturais do parasita e servem como fonte de infecção para o vetor. O cão pode ser apontado como o principal reservatório doméstico para a LV. Raposas e lobos são reservatórios silvestres da LV, enquanto marsupiais e roedores podem ser reservatórios de espécies que causam a LT. Os vetores são flebotomíneos, fêmeas (Díptera: Psicodidae), pertencentes ao gênero *Lutzomya* nas Américas e *Phlebotomus* em países do Velho Mundo (Sacks & Kamhawi, 2001).

A infecção pelo vetor pode ocorrer no momento em que a fêmea se alimenta de um hospedeiro infectado quando, juntamente com o sangue, pode ingerir células fagocíticas contendo formas amastigotas do parasita. Ocorre, então, a liberação das formas amastigotas no tubo digestivo do vetor que, rapidamente, passam por mudanças bioquímicas e morfológicas e evoluem para a forma promastigota procíclica e, então, à promastigota metacíclica. O hospedeiro mamífero é infectado quando é picado por um vetor contaminado; quando o mesmo pode injetar ou regurgitar formas promastigotas metacíclicas na derme do hospedeiro. Os parasitas podem ser opsonizados por proteínas do sistema do complemento ou anticorpos e fagocitados por macrófagos, formando os fagolisossomos**,** onde se transformam em formas amastigotas. Após sucessivas replicações das amastigotas, essas podem causar a lise dos macrófagos com consequente liberação das mesmas. As amastigotas podem ser fagocitadas por novos macrófagos ou outras células fagocíticas, finalizando o ciclo de infecção no hospedeiro mamífero, ou podem ser ingeridas por outro vetor, completando, assim, o ciclo biológico do parasita (Figura 3).

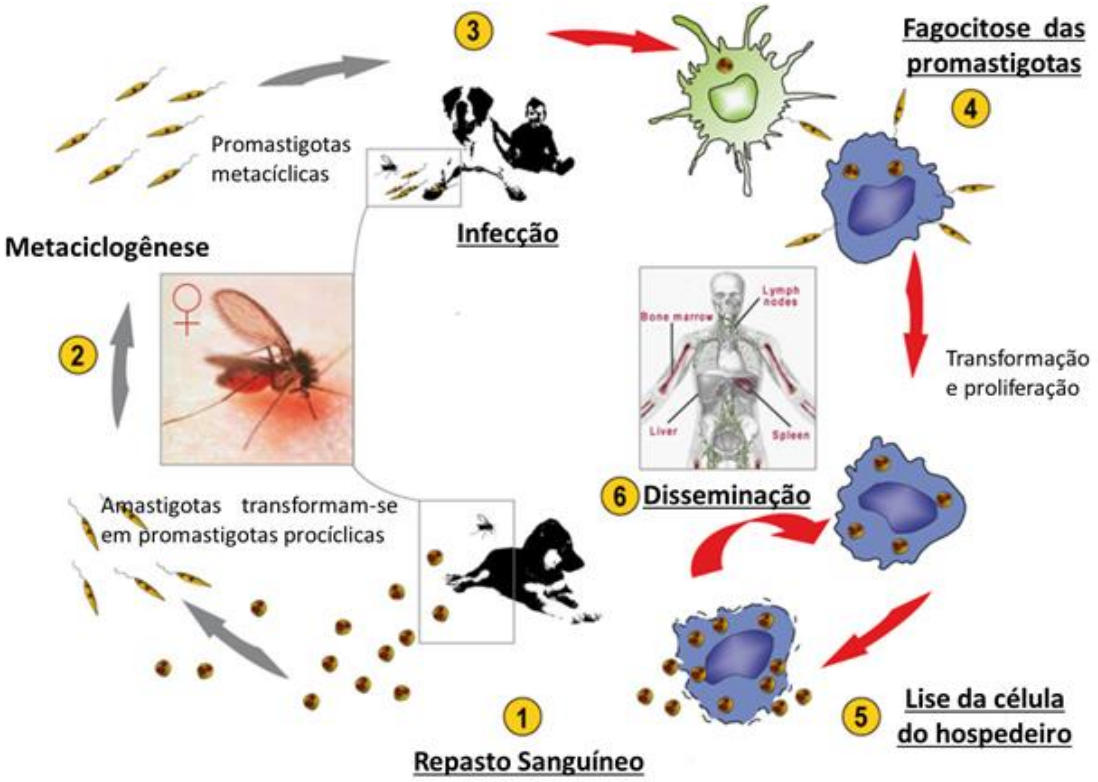

Figura 3: Ciclo biológico do parasita *Leishmania.* 1. Infecção do vetor no momento em que realiza o repasto sanguíneo em um hospedeiro infectado (ingestão de amastigotas). 2. Transformação das formas amastigotas em promastigotas metacíclicas no interior do vetor. 3. Inoculação das formas promastigotas metacíclicas e infecção no hospedeiro mamífero. 4. Fagocitose dos parasitas por macrófagos, transformação em amastigotas e proliferação. 5. Lise da célula hospedeira com liberação de amastigotas. 6. Disseminação dos parasitas para órgãos e tecidos do hospedeiro mamífero. Adaptado de Nieto et al. (2011).

2.3. Manifestações clínicas das leishmanioses

Diferentes espécies parecem estar associadas a diferentes quadros clínicos das leishmanioses. A LT caracteriza-se pela diversidade de manifestações clínicas e de espécies causadoras da doença. Está associada a infecções por espécies como *L. major* e *L. tropica* no Velho Mundo e a uma diversidade de espécies nas Américas. No Brasil, a LT pode ocorrer devido à infecção por *L. braziliensis, L. guyanensis, L. amazonensis, L. shawi, L. laisoni* e *L. naiffi* podendo, clinicamente, apresentar-se de forma localizada ou disseminada. A leishmaniose cutânea (LC) caracteriza-se pela existência de uma lesão única com bordas

elevadas, de fundo granuloso e indolor. Lesões vegetantes, verrucosas ou infiltrativas são menos frequentes (Marzochi et al., 1994; Desjeux, 2004; Silveira et al., 2004).

Casos de leishmaniose muco-cutânea (LMC) ocorrem em diversos países e são causadas pelas espécies *L. braziliensis, L. panamensis, L. guyanensis* e *L. amazonensis*. As lesões apresentam caráter infiltrativo, que podem ulcerar e sangrar. Embora a LMC seja mais frequentemente encontrada nas Américas, casos de LMC têm sido relacionados à infecção com *L. major* e *L. donovani* em países do Velho Mundo (Desjeux, 2004).

A forma cutâneo-difusa (LCD) da doença ocorre devido a infecção pela espécie *L. aethiopica*, existente na África ou pelas espécies *L. amazonensis* e *L. mexicana* nas Américas. É uma forma em que as lesões podem apresentar-se como placas, nódulos, às vezes vegetantes, mas que raramente ulceram. As lesões disseminam-se em regiões expostas do corpo e tal quadro pode estar associado à ineficiência ou ausência de resposta celular (Weigle & Saraiva, 1996; Desjeux, 2004). A LCD, embora rara, não apresenta cura espontânea. Devido às frequentes recidivas, tornou-se um grave problema de Saúde Pública, juntamente com as demais formas de LT, pois, além da elevada incidência e ampla distribuição geográfica, o indivíduo infectado pode apresentar lesões destrutivas, desfigurantes e incapacitantes, excluindo-o do seu meio de vida social (Gontijo & Carvalho, 2003; Desjeux, 2004).

A LV ocorre pela infecção por *L. donovani* e *L. infantum* em países do Velho Mundo e pela infecção por *L. chagasi* nas Américas (Lainson et al., 1985). Recentemente, alguns trabalhos têm sugerido que as denominações *L. chagasi* e *L. infantum* podem se referir à mesma espécie, devendo ser apontadas como sinônimos (Maurício et al., 2000). A infecção pode tornar-se crônica e causar febre irregular de longa duração, hepatoesplenomegalia, linfadenopatia, anemia, leucopenia, edema, debilidade progressiva e emagracimento, podendo levar à morte na ausência de tratamento. A intensidade das manifestações clínicas é variável e indivíduos podem permanecer assintomáticos, embora cerca de 20% dos infectados possam desenvolver a doença em sua forma clássica. Os sintomas são progressivos e as complicações decorrentes da evolução da infecção são responsáveis pela maioria dos óbitos (Badaró et al., 1986; Gama et al., 2004).

Casos de LV causada pela espécie *L. amazonensis* foram descritos na Bahia (Barral et al., 1991). A LV tornou-se uma importante doença oportunista em indivíduos infectados com o HIV, especialmente em países da Europa (Lopes-Velez et al., 1998).

Epidemiologicamente, a leishmaniose visceral canina (LVC) apresenta-se com maior grau de importância quando comparada à doença humana, devido ao maior registro de casos da doença canina comparada à doença humana (Barão et al., 2007; Queiroz et al., 2008). A LVC pode apresentar manifestações clínicas que variam consideravelmente, dependendo de fatores como a interação com a espécie do parasita, a fase atual da doença e a resposta imune do animal. O período de incubação da doença pode variar de 1 mês a 4 anos, e cães infectados podem também permanecer assintomáticos por um longo período de tempo (Lanotte et al., 1979; Keenan et al., 1984). Neste caso, ainda que aparentemente saudáveis, comportam-se como reservatórios do parasita e fonte de transmissão do mesmo em relação aos flebotomíneos e o homem (Verçosa et al., 2008).

2.3. Tratamento das leishmanioses

O tratamento das leishmanioses compreende o uso de antimoniais pentavalentes, tais como o antimoniato de N-metil glucamina, produzido sob o nome comercial de Glucantime→ (Rhône Poulenc Rorer, França) e o estibogluconato de sódio, produzido sob o nome comercial de Pentostan→ (Welcome Foundation, Inglaterra) (Figueiredo et al., 1999).

No Brasil, o Glucantime→ tem sido utilizado como fármaco de escolha, entretanto, os antimoniais, de modo geral, interagem com sulfidrilas de proteínas celulares causando perda de função e/ou formando complexos com ribonucleosídeos (Demecheli et al. 2002), o que induz à inespecificidade de ação do produto frente às células infectadas e não-infectadas. Fármacos de segunda linha tais como a anfotericina B têm sido recomendados nos casos de intolerância ou resistência ao tratamento convencional, devendo ser administrados exclusivamente em ambiente hospitalar (Sundar et al., 2009).

O tratamento com antimoniais pentavalentes possui diversas limitações que reduzem a adesão do paciente ao mesmo. Podem ser citadas as vias de aplicação dos fármacos (intramuscular ou endovenosa) e os efeitos colaterais graves, tais como toxicidade renal, hepática e cardíaca gerando arritmias e alterações eletrocardiográficas. Soma-se ao fato de que a eficácia do tratamento depende da espécie de *Leishmania* que está causando a doença (Tuon et al., 2008).

Apesar de existirem alternativas de tratamento tanto para a LV (tabela 1) quanto para a LT (Tabela 2), nenhum dos medicamentos é capaz de eliminar o parasita. Além disso,

produzem inúmeros efeitos colaterais (Tabela 1 e 2). Outro fator que dificulta a disponibilização de um tratamento de qualidade para os pacientes são os preços elevados dos mesmos (Tabelas 3 e 4), que podem variar de R$ 203,00 (tratamento com antimônio) até R$ 917,00 (tratamento com anfotericina B lipossomal). Ainda que em nosso país o tratamento seja custeado pelo governo, os valores elevados dos produtos acabvam por sobrecarregar as contas públicas.

Tabela 1: Sumário de opções para o tratamento de Leishmaniose visceral.

Fonte: Adaptado de E. M. Moor, D. N. Lockwood 2010.

Terapias para LV	Vantagens	Desvantagens	Lugares utilizados
Estibugluconato de Sódio (SSG)	Longo histórico de eficácia (mesmo em circunstâncias de dificuldade)	Toxicidade (vômitos, cardiáca e fígado) Falha em tratamentos na Índia Tratamento longo Sem preparação oral	Leste da África
Anfotericina B	Boa eficácia, especialmente em tratamentos em que há falha pelo SSG	Toxicidade (reações a infusões, renal) Tratamento longo Sem preparação oral	Índia
Anfotericina B Liposomal	Ecelente eficácia, mesmo para pacientes HIV+ Tratamento curto	Alto custo Sem preparação oral	Países desenvolvidos Índia
Miltefosina	Preparação oral	Toxicidade reprodutiva Toxicidades (gastrointestinal)? pouco efetiva (mas menos letal) comparada	Índia

		com SSG em pacientes HIV+	
Paramomicina	Barata Taxas de cura equivalentes a anfotericina B na Índia (provavelmente não na África) Amplo espectro de atividade tornando útil em grupos intercorrentes de diarréia na África	Suprimento variável da droga Toxicidade (citotóxica, fígado) Sem preparação oral	Índia Em combinação com SSG na África
Pentamidina	Útil como prevenção secundária para pacientes HIV+	Baixa eficácia como tratamento primário Toxicidade (cardíaca, diabetes, gastrointestinal)	América do Sul Profilaxia para pacientes HIV+ na europa

Por tais fatos, é comum o abandono do paciente ao tratamento ou a interrupção do mesmo, o que leva ao aumento da resistência do parasita aos fármacos. Há de se destacar ainda o aumento de casos de recidivas à doença nos pacientes após o tratamento, fato atualmente observado em diversas regiões do mundo (Vélez et al., 2009).

Outro fator que pode estar relacionado com a pouca oferta de tratamentos específicos para as leishmanioses são as limitações técnicas para os estudos pré-clínicos nos diversos modelos experimentais da doença. As técnicas convencionais são laboriosas e demandam tempo, além do fato de que os modelos pré-clinicos se tornam quase inviáveis para serem confeccionados de maneira desejada, uma vez que é preciso eutaniziar vários grupos de animais para cada tempo de tratamento e para cada dose diferente. Isso é mais problemático nos modelos viscerais, como para *L. chagasi*, em que os testes pré-clinicos são uma incógnita sem nenhum dado experimental fidedigno até o momento da eutanásia dos animais. Metodologias como o imagemaneto *in vivo* de parasitas, têm se mostrado uma ferramenta promissora para os testes pré-clínicos em modelos de leishmanioses, pois nessa técnica se utiliza o mesmo grupo de animais ao longo do tratamento e experimento, sem a necessidade de eutanásia dos animais, o que acaba por reduzir o número de animais utilizados, permitindo um estudo longitudinal dos mesmos e levando à uma maior flexibilidade na análise do tempo e tratamento necessários (Talhofer et al. 2010, Mehat et al. 2008).

Tais fatos mostram a necessidade de estudos objetivando o desenvolvimento de novos compostos ou a utilização daqueles já existentes em novos esquemas terapêuticos, como uma melhor via de administração dos fármacos, a fim de garantir a adesão dos pacientes ao tratamento completo e possibilitar o sucesso do tratamento (Romero et al., 2001).

Tabela 2: Sumário de opções para o tratamento de Leishmaniose tegumentar.

Fonte: Adaptado de M. Ameen, 2110

	Regime de Tratamento	Espécie	Resposta terapêutica (% de cura)	Efeitos Adversos
Terapia local				
Antimônio intralesão	Infiltração da lesão até a base Terapia semanal até a cura	*L. major* *L. tropica* *L. mexicana* complex	Até 95%	Dor no local da injeção
Paramomicina tópica in 10% de uréia ou 12% cloreto de metilbenzetônio	Aplicação duas vezes por dia por até 4 semanas Pode ser combinada com antimoniais intralesão	*L. major* *L. mexicana complex*	Altamente variável 30-90%	Irritação local
Crioterapia	Tratamento semanal de dois ciclos de congelamento e descongelamento por 10-25s até a cura Mais indicada e efetiva para pequenas lesões	*L. major* *L. tropica* *L. mexicana complex*	70-80% Mais alta quando combinada com antimoniais intralesão ou paramomicina tópica	Risco de infecção secundária e despigmentação pós-inflamatória
Termoterapia	1-3 aplicações de ondas de rádio-frequência de 50C° por 30 s Uso depende do tamanho da lesão e número de lesões	*L. tropica* *L. mexicana complex*	70%	Usualmente bem tolerada
Quimioterapia oral				
Azóis	Fluconazol 200mg por dia por 6 semanas Cetoconazol 600mg por dia por 4-6 semanas	*L. major* *L. mexicana complex*	70-80% Geralmente melhor responsta com Fluconazol	Altas doses de cetoconazol aumenta o risco de hepatotoxicidade
Miltefosina	2,5mg/kg/dia por 20-28 dias	*L. panamensis*	> 90%	Distúrbios gastrointestinais, teratogênico
Quimioterapia parenteral				
Antimoniais (Stibogluconato de sódio ou antimoniato de meglumina)	20mg/kg/dia por 20-28 dias	*L. brasiliensis complex*	> 90%	Cardiotóxico, hapatotóxico, nefrotóxico
Isetionato de Pentamidina	Três doses em dias alternados até 2-4mg/kg	*L. brasiliensis complex*	> 90%	Pode induzir diabetes melitus
Anfotericina	Segunda linha de agente para infecções severas 0,5-1mg/kg em dias alternados por até 8 semanas (dose total <1.5-2g)	*L. brasiliensis complex*	Apenas estudo de casos	Nefrptoxicidade Preparação liposomal menos tóxica

Tabela 3: Custos de tratamentos para Leishmaniose visceral. Fonte: WHO, 2010.

Composto	**Regime de tratamento**	**Custo da droga em US$[a]**
L-Amb 10 mg/kg	1 dia	126
L-Amb 20 mg/kg	2-4 dias	252
Anfotericiana B deoxicolato 1 mg/kg (dias alternados)	30 dias	20
MF 100 mg/kg/dia	28 dias	65 - 150
PM 15 mg/kg/dia	21 dias	15
SSG 20 mg/kg/dia	30 dias	55,8
MA 20 mg/kg/dia	30 dias	59,3
L-Amb 5 mg/kg + MF 100 mg/dia	8 dias	88,2 - 109,5
L-Amb 5 mg/kg + PM 15 mg/kg/dia	11 dias	79
MF 100 mg/dia + PM 15 mg/kg/dia	10 dias	30,2 – 60,7
(SSG 20 mg + PM 15 mg)/kg/dia	17 dias	44

[a] Para paciente pesando 35 kg. Cálculos para SSG e MF baseados na taxa de
€ 1= US$ 1,41 (28 janeiro 2010). Variação de preço para miltefosina depende
Do volume do pedido.
Preço baseado em SSG genérico.
L-Amb = Anfotericina B lipossomal, MF = Miltefosina, PM = Paramomicina
SSG = Estibogluconato de sódio, MA = Antimoniato de meglumina

Tabela 4: Custos de tratamentos para Leishmaniose tegumentar. Fonte: WHO, 2010.

Composto	Regime de tratamento	Custo da droga em US$[a]
SSG sistêmico, 20 mg/kg/dia	20 dias	37,2
SSG intralesão [b]	Até cicatrização da lesão	12
MA sistêmico, 20 mg/kg/dia	20 dias	39,5
MA intralesão [b]	Até cicatrização da lesão	13,2
Pentamidina	Até 4 meses	Gratuito (programa de doação)

[a] Para paciente pesando 35 kg. Cálculos para SSG e MF baseados na taxa de € 1= US$ 1,41 (28 janeiro 2010). Preço baseado em SSG genérico.

[b] Tratamento intralesão é comumente estimado até um terço do custo do tratamento sistêmico.

SSG = Estibogluconato de sódio, MA = Antimoniato de meglumina

Pelos fatos expostos verifica-se que além de ser necessária a busca de novos tratamentos paras as diversas formas clínicas de leishmanioses, hipotetizamos que o fungo *Agaricus blazei* pode ter o potencial para tal fim, uma vez que apresenta propriedades antimicrobianas (Sakagami et al., 1991) e imunomodulatórias (Mizuno et al. 2013). Entretanto, até o presente momento, nenhum estudo *in vitro* ou *in vivo* mostrou essa aplicação. Assim, a tese apresentada tem como objetivo investigar o possível potencial terapêutico do cogumelo *Agaricus blazei* no tratamento de leishmanioses.

3. Objetivo Geral

Verificar a atividade leishmanicida do fungo *Agaricus blazei*, em experimentos *in vitro* e *in vivo,* contra diferentes espécies de *Leishmania*, no caso, *L. amazonensis, L. major* e *L. chagasi*, na tentativa de avaliar o potencial terapêutico desse cogumelo para o seu emprego no tratamento das leishmanioses.

3.1 Objetivos específicos

A) Verificar a atividade leishmanicida do *Agaricus blazei* em diferentes espécies de leishmania, *in vitro*

B) Avaliar a eficácia do *Agaricus blazei* no tratamento da infecção causada pela espécie *L amazonensis* em camundongos BALB/c.

C) Avaliar a eficácia do extrato aquoso e de frações do *Agaricus blazei* no tratamento e prevenção (quimioprofilaxia) da infecção causada pela espécie *L. chagasi* em camundongos BALB/c.

D) Envolvimento de Nlrp12 (nucleotide-binding domain leucine-rich repeat protein 12) na leishmaniose visceral.

Cada um dos objetivos específicos refere-se a um trabalho publicado ou em preparação que serão apresentados a seguir.

Parte 2

TRABALHO EXPERIMENTAL

CAPÍTULO 1

Leishmanicidal activity of the *Agaricus blazei* Murill in different *Leishmania* species. Parasitology International, volume 60, número 4, p. 357–363, 2011.

1º objetivo da tese: Na primeira parte do trabalho foi proposta a preparação do extrato aquoso do fungo *Agaricus blazei* e sua avaliação em atividade leishmanicida, por meio de experimentos *in vitro*, contra as espécies *L. amazonensis*, *L. major* e *L. chagasi*. A citotoxicidade do extrato aquoso foi avaliada em macrófagos murinos e hemáceas humanas. Uma série de experimentos foram conduzidos e os resultados obtidos permitiram a publicação do artigo científico intitulado "Leishmanicidal activity of the *Agaricus blazei* Murill in different *Leishmania* species", que será apresentado a seguir, em sua íntegra.

Título do artigo:

Leishmanicidal activity of the *Agaricus blazei* Murill in different *Leishmania* species.

Referência no PUBMED:

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ABSTRACT

Leishmaniasis is a major public health problem, and the alarming spread of parasite resistance underlines the importance of discovering new therapeutic products. The present study aims to investigate the *in vitro* leishmanicidal activity of an *Agaricus blazei* Murill mushroom extract as compared to different *Leishmania* species and stages. A water extract proved to be effective against promastigotes and amastigotes-like stages of *Leishmania amazonensis*, *L. chagasi*, and *L. major*, with IC_{50} values of 67.5, 65.8, and 56.8 μg/mL for promastigotes, and 115.4, 112.3, and 108.4 μg/mL for amastigotes-like stages, respectively. The infectivity of the three *Leishmania* species before and after treatment with the water extract was analyzed, and it could be observed that 82%, 57%, and 73% of the macrophages were infected with *L. amazonensis*, *L. major*, and *L. chagasi*, respectively. However, when parasites were pre-incubated with the water extract, and later used to infect macrophages, they were able to infect only 12.7%, 24.5%, and 19.7% of the macrophages, presenting reductions in the percentage of infections in the order of 44.1%, 30.1%, and 45.2% for *L. amazonensis*, *L. chagasi*, and *L. major*, respectively. In others experiments, macrophages that were infected with *L. amazonensis*, *L. chagasi*, or *L. major*, and later treated with the aforementioned extract, presented reductions of 84.4%, 79.6%, and 85.3% in the parasite burden after treatment. A confocal microscopy revealed the lost of the viability of the parasites within the infected macrophages after treatment with the water extract. The applied extract presented a low citotoxicity in murine macrophages and a low hemolytic activity in type O^+ human red blood cells. No nitric oxide (NO) production, nor inducible nitric oxide syntase expression, could be observed in macrophages after stimulation with the water extract, suggesting that biological activity may be due to direct mechanisms other than macrophage activation by means of NO production. In conclusion, the results demonstrate that the *A. blazei* Murill water extract can potentially be used as a therapeutic alternative on its own, or in association with other drugs, to treat visceral and cutaneous leishmaniasis.

KEYWORDS: *Leishmania, Agaricus blazei* Murill Mushroom, Leishmanicidal Activity, Treatment.

1. Introduction

Protozoan parasites of the *Leishmania* genus are the etiological agents of a vector-borne disease that has presented high morbidity and mortality throughout the world. Leishmaniasis has affected around 12 million people and is present in 88 countries, mainly in tropical and subtropical regions. The approximately 2 million new cases per year and the nearly 350 million people living in endemic regions reveal the importance of this neglected disease [1,2].

Historically, chemotherapy to treat leishmaniasis has been based on the use of pentavalent antimonial drugs. One of the available anti-leishmanial drugs, sodium antimony gluconate, is clinically unsatisfactory, given that many visceral leishmaniasis (VL) cases show no response to it. Furthermore, the cases that do respond to this form of treatment tend to relapse at a later stage [3,4]. Pentamidine, another anti-leishmanial drug, is unsuitable as a first line treatment due to its toxicity. Amphotericin B and its liposomal formulation are effective, though such drugs are expensive and their use requires hospitalization. Reported clinical results using oral miltefosine treatment are encouraging; however, this drug is linked to potential teratogenicity and should not be given to pregnant women or to those of childbearing age [5]. The number of reported VL cases is increasing. Moreover, VL has emerged as an opportunistic infection in human immunodeficiency virus-infected (HIV) patients [6]. Therefore, the development of cost-effective alternative therapeutic strategies has become a high-priority.

Prior research has been carried out concerning natural products and their biological effects, such as fungicidal, antimicrobial, antimalarial, antimycobacterial, and antiviral activities. However, only a few studies have investigated the biological potential of Brazilian mushrooms. *Agaricus blazei* Murill is a commonly found mushroom in Brazil. Its use has been associated with folk medicine in the treatment of some diseases, including diabetes and arterial hypertension [7-13]. This mushroom presents compounds, such as β-D-glucans, glycoproteins, cerebrosides, polysaccharides, steroids, ergosterol, and graxs acids, which can activate and/or modulate the immune response of the hosts [14-17].

The present study, therefore, assessed the *in vitro* leishmanicidal activity of an *A. blazei* Murill water extract against *L. amazonensis*, *L. chagasi*, and *L. major* promastigote and amastigote-like stages. Studies were extended to establish their minimum inhibitory concentrations (IC_{50}), their leishmanicidal effects on intra-macrophage *Leishmania* stages,

nitric oxide (NO) production, inducible nitric oxide syntase (iNOS) expression, as well as their cytotoxic effects on murine macrophages and human red blood cells.

2. Materials and methods

2.1. *Agaricus blazei* Murill water extract

The *Agaricus blazei* Murill water extract was prepared by macerating 50g of fresh mushrooms in 50mL of sterile milli-Q water using a Waring-Blendor homogenizer. Next, the macerated mixture was centrifuged at 10.000 x *g* for 20 min at 4°C (Sorval, LC5C model). The supernatant was then sterilized by passing through a 0.22 µm membrane in a laminar flow hood under sterile conditions and stored at −20°C until use. This procedure has been patented at the Federal University of Minas Gerais (UFMG) (PI 014100001550/CT&T).

2.2. Parasites

Leishmania amazonensis (IFLA/BR/1967/PH-8), *L. chagasi* (MHOM/BR/1970/BH46), and *L. major* (MHOM/IL/1980/Friedlin) were used in this study. Parasites were grown at 24ºC in Schneider's medium (Sigma, St. Louis, MO, USA), supplemented with 20% heat-inactivated fetal bovine serum (FBS, Sigma), 20 mM L-glutamine, 200 U/mL penicillin, and 100 µg/mL streptomycin, at a 7.4 pH [18]. *Leishmania* stationary-phase promastigote and amastigote-like stages used in this work were prepared as described [19].

2.3. Leishmanicidal *in vitro* activity

Inhibition of cell growth was assessed *in vitro* by cultivating promastigote and amastigote-like ($4x10^5$, each one) stages of *L. amazonensis*, *L. chagasi*, and *L. major* in the presence of different individual concentrations (25 to 200 µg/mL) of *A. blazei* Murill water extract in 96-well culture plates (Corning Life Sciences, Corning, NY, USA), for 48 h at 24ºC. Cell viability was assessed by measuring the cleavage of 2 mg/ml of MTT [3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide] (Sigma). Absorbances were measured by using a multi-well scanning spectrophotometer (LABTRADE, model 660) at a wavelength of 570 nm. Analyses were performed in triplicate, and results were expressed as the mean percentage reduction of the parasites compared to non-treated control wells. The 50%

inhibitory concentration (IC_{50}) was determined by applying the sigmoidal regression of the concentration-response curves. Data shown are representative of three different experiments, performed in triplicate, which presented similar results.

2.4. Chemical characterization of the *Agaricus blazei* Murill water extract

Chemical analysis of the water extract was performed on chromatography plates coated with silica gel GF 254® (Merck, Darmstadt, Germany) for detection of tannins, coumarins, flavonoids, anthraquinones, triterpenes, steroids, saponins, cardiotonic glycosides, and alkaloids. Different mobile phases and detection reagents were used in accordance with the protocol described by Wagner et al. (1984) [20]. Proteins were detected in SDS-PAGE 10% gels by silver staining, while glycoproteins presence were demonstrated in SDS-PAGE 10% stained by periodic acid Schiff. The presence of carbohydrates was investigated using a phenol–sulfuric acid method [21].

2.5. Inhibition of infection in phagocytic cells

The inhibitory effect of the *A. blazei* Murill water extract on the *Leishmania* invasion of macrophages was evaluated in promastigotes of *L. amazonensis, L. chagasi*, and *L. major*. Parasites were pre-incubated with the water extract (25 µg/mL), for 1 h at 24ºC. Next, cells were washed three times with RPMI 1640 medium and further incubated for 4 h with murine macrophages at a ratio of 10 *Leishmanias* per 1 macrophage. After, cells were again washed, set, and stained, to determine the percentages of infected macrophages by counting 100 cells in triplicate. Additionally, an optical microscopy was used to view the *Leishmania* infection profiles within the murine macrophages.

2.6. Treatment of infected macrophages

Macrophages were plated on round glass coverslips inside the wells of a 24-well culture plate at a concentration of $5x10^5$ cells per coverslip in an RPMI 1640 medium supplemented with 20% FBS, 2 mM L-glutamine, 200 U/mL penicillin, and 100 µg/mL streptomycin, at a 7.4 pH. After 2 h of incubation at 37°C in 5% CO_2; promastigotes of *L. chagasi*, *L. major*, and *L. amazonensis* were added to the wells ($5x10^6$), and the cultures were incubated for 4 h at 37°C, 5% CO_2. Next, free parasites were removed by extensive washing with an RPMI 1640

medium, and infected macrophages were treated for 48 h with the *A. blazei* Murill water extract (50 μg/mL). Cells were washed in RPMI 1640 and incubated with 4% paraformaldehyde for 15 min, at which time they were treated with 70% ethanol in an ice-bath for 4 h and again washed three times with sterile PBS. The percentage of the inhibition of *Leishmania* intra-macrophage viability was determined by counting 100 cells in triplicate and comparing this to the infected and non-treated cells. Additionally, a confocal microscopy was performed to view the inhibition of *Leishmania* viability within intra-macrophages. For this, an RNaseA solution was added (200 μg/mL), and incubation occurred for 30 min at 37°C, after which time, a solution comprised of 370 μL of 0.1 M HCl in 148 μL of PBS 1x was added per well. After 45 seconds, 1.5 mL of an acridine orange solution (3 μg/mL, pH 5.0) was added and incubation occurred for 5 min, at which time the cells were analyzed. Data shown are representative of three separate experiments, performed in triplicate, which presented similar results.

2.7. Nitric oxide (NO) production and iNOS Western blot

To visualize the macrophage activation via NO production after treatment with an *A. blazei* Murill water extract, cells ($4x10^5$) were incubated alone in an RPMI 1640 medium (background control) or separately stimulated with the water extract (50 μg/mL) or concanavalin A (ConA; 5 μg/mL), at 37^0C in 5% CO_2 for 24, 48, and 72 h. Following incubation, 100 μl of culture supernatant was mixed with an equal volume of Griess reagent (Sigma). After an incubation of 30 min at room temperature, nitrite concentration was calculated using a standard curve of known concentrations. Data were expressed as μM per $4x10^5$ cells. Data shown are representative of three different experiments, performed in triplicate, which presented similar results.

After separation of the supernatants, and to detect iNOS expression, cells were lysed in an ice-cold lyses buffer (100 mM Tris; pH 8; 2 mM EDTA; 100 mM NaCl; 1% Triton X-100), which was supplemented by a complete proteases inhibitors cocktail (Roche Diagnostics, Indianapolis, IN, USA). Lysates were centrifuged at 14,000 x *g* for 10 min at 4°C, and the protein concentration was determined using the DC Protein Assay Reagents Package (Bio-Rad Laboratories, Hercules, CA, USA), employing bovine serum albumin (BSA) as a standard. The proteic extract (20 μg) was analyzed by means of a 12%SDS–PAGE, and proteins were transferred onto cellulose membranes using a semi-dry electroblotting apparatus (Loccus

Biotecnology). The membranes were blocked with a 5% BSA in Tris saline-Tween 20 (TBS-T: 10 mM Tris–HCl; pH 7.4; 0.15 M NaCl; 0.05% Tween 20), for 2 h at room temperature. Blots were incubated with 1:1000 monoclonal anti-mouse iNOS (123 kDa) and beta-actin (45 kDa) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight, under constant shaking, at 4°C. After having been washed 5 times with TBS-T, the membranes were incubated using an anti-mouse IgG goat antibody (Cell Signaling Technology, Danvers, MA, USA, dilution 1:1000 in 1% non-fat dry milk) for 2 h at room temperature. After having been washed 7 times with TBS-T, blots were developed using cloronaftol (12.5 mg), diaminobenzidine (25 mg), and H_2O_2 30 vol. (20 µL), for 30 min, and the reaction development was ended by adding distilled water.

2.8. Citotoxicity and hemolytic activity

Citotoxicity was evaluated by cultivating macrophages ($4x10^5$) with different concentrations (25 to 200 µg/mL) of the *A. blazei* Murill water extract in 96-well plates, for 24 h. Cell viability was assessed by the MTT assay, and results were expressed as the percentage of cell lyses compared to cultures treated with amphotericin B (1 µg/mL). The hemolytic activity was investigated by incubating the *A. blazei* Murill water extract (25 to 200 µg/mL) with a 5% red blood cell (human O^+) suspension for 1 h at 37ºC. The erythrocyte suspension was centrifuged (1000 x *g* for 10 min), and cell lysis was determined spectrophotometrically (540 nm), as described [22]. The absence of (blank) or 100% presence of hemolysis (positive control) were determined by replacing the extract for an equal volume of PBS or distilled water, respectively. The results were determined by the percentage of hemolysis compared to the negative and positive controls. Data shown are representative of three separate experiments, performed in triplicate, which presented similar results.

2.9. Statistical analysis

Statistical analysis was performed using the GraphPad Prism software (version 5.0 for Windows). The differences among the diverse treatments (25, 50, 100 or 200 µg/mL) carried out using the *A. blazei* Murill water extract, as well as the reduction of the percentage of infected macrophages, were evaluated by the one-way ANOVA analysis, followed by

Bonferroni's post-test for multiple comparisons. Differences were considered significant when $P < 0.05$.

3. RESULTS

3.1. Leishmanicidal activity of the water extract of *A. blazei* Murill in *Leishmania*

The *in vitro* leishmanicidal effect of the *A. blazei* Murill water extract was tested against promastigote and amastigote-like stages of *L. amazonensis, L. chagasi*, and *L. major*. Parasites were separately incubated with different extract concentrations (25 to 200 μg/mL) for 48 h at 24 °C. In the evaluation of the results, it could be observed that the leishmanicidal activity was based on a dependent-dose application, given that that a lower dose (25 μg/mL) of the extract induced the inhibition of Leishmania viability percentages in the order of 26.5%, 29.3%, and 23.2% for *L. amazonensis, L. chagasi*, and *L. major* promastigotes (Fig. 1), respectively, whereas in the amastigote-like stage, the inhibition of cell viability percentages were of 10.5%, 12.3%, and 13.2%, respectively (Fig. 2) The higher dose of the water extract (200 μg/mL) evoked higher inhibition of *Leishmania* viability percentages in the order of 85%, 81.6%, and 85% for *L. amazonensis, L. chagasi,* and *L. major* promastigotes, respectively, and of 61.5%, 63.6%, and 65.6% for amastigote-like stages, respectively, for the three species. In the evaluation of IC50, promastigotes proved to be more susceptible than amastigote-like as they presented IC50 values of 67.5, 65.8, and 56.8μg/mL for *L. amazonensis, L. chagasi*, and *L. major*, respectively, while the values for amastigote-like stages were 115.4, 112.3, and 108.4 μg/mL for *L. amazonensis, L. chagasi*, and *L. major*, respectively (Table 1). Amphotericin B (10 μg/mL), used as a positive control, presented significant reductions in the viability of the three *Leishmania* species evaluated (Figs. 1 and 2).

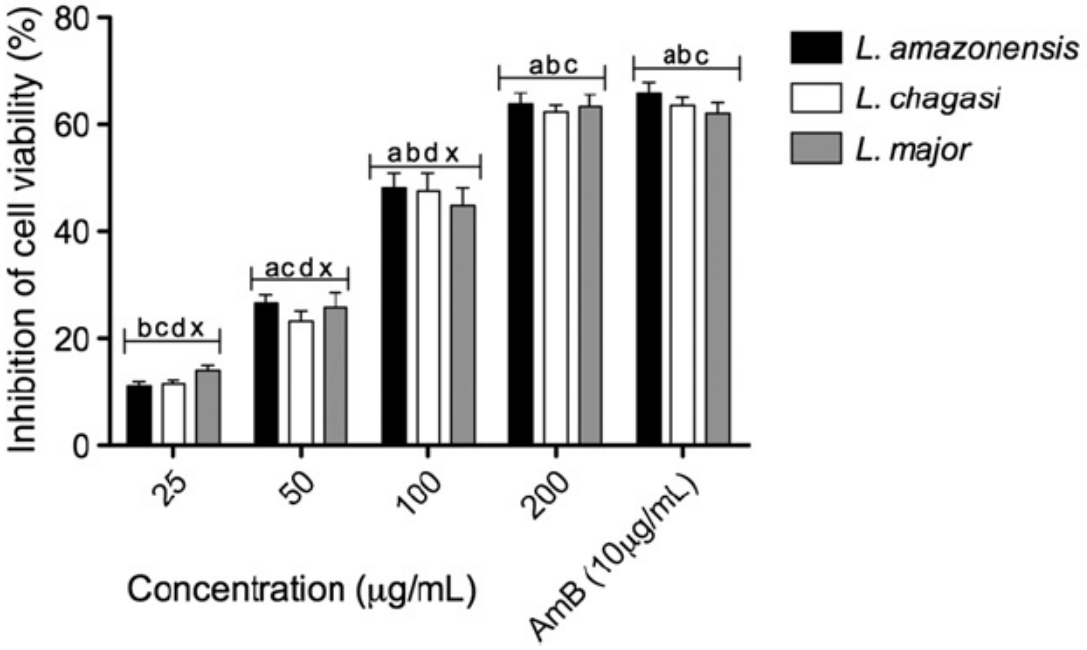

Figura 1- Leishmanicidal activity of the water extract of *Agaricus blazei* Murill against promastigotes of L. chagasi, L. major, and L. amazonensis. Parasites (4 × 105) were incubated with different individual concentrations (25 to 200 µg/mL) of the A. blazei Murill water extract, and cell viability was analyzed by MTT assay, after incubation for 48 h at 24 °C. Amphotericin B (AmB, 10 µg/mL) was used as a positive control. Bars indicate the mean ± standard deviation of the inhibition of Leishmania viability in each concentration tested. Differences were considered statistically significant (Pb0.05) when comparing the parasites treated with: (a) 25 µg/mL, (b) 50 µg/mL, (c) 100 µg/mL, or (d) 200 µg/mL of the water extract, and the parasites treated with AmB (x).

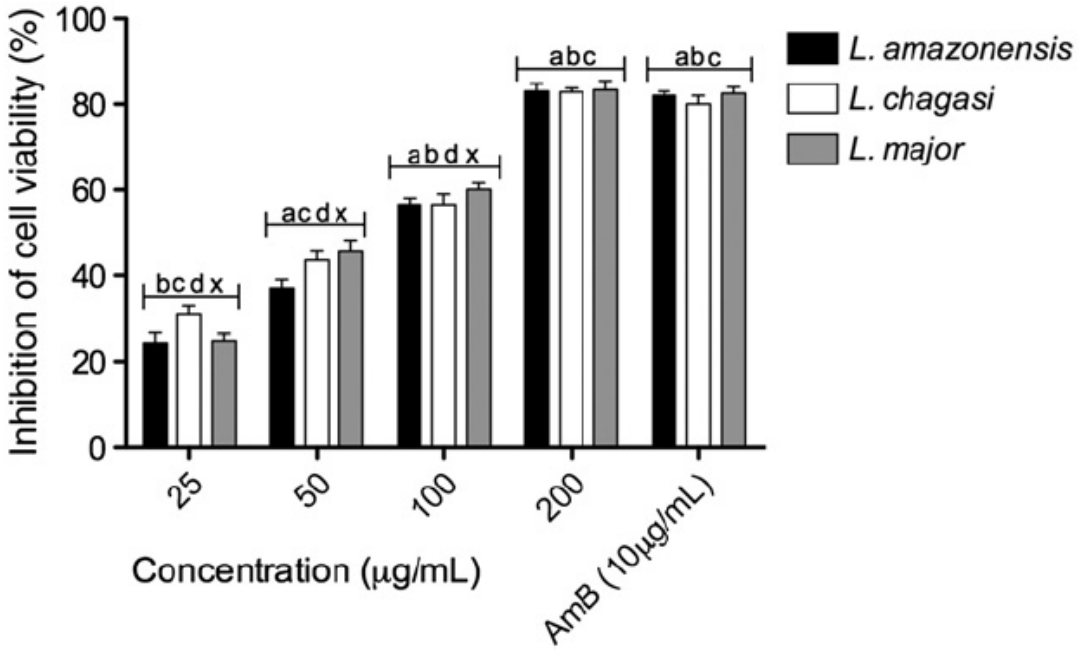

Figura 2- Leishmanicidal activity of the water extract of *Agaricus blazei* Murill against amastigotes-like of *L. chagasi*, *L. major*, and *L. amazonensis*. Parasites (4 × 105) were incubated with different individual concentrations (25 to 200 μg/mL) of the *A. blazei* Murill water extract, and cell viability was analyzed by MTT assay, after incubation for 48 h at 24 °C. Amphotericin B (AmB, 10 μg/mL) was used as a positive control. Bars indicate the mean ± standard deviation of the inhibition of *Leishmania* viability in each concentration tested. Differences were considered statistically significant (P b 0.05) when comparing the parasites treated with: (a) 25 μg/mL, (b) 50 μg/mL, (c) 100 μg/mL, or (d) 200 μg/mL of the water extract, and the parasites treated with AmB (x).

Tabela 1 - Fifty percent inhibitory concentration (IC50) of the water extract of *Agaricus blazei* Murill against different *Leishmania* species. The results are expressed as mean± standard deviation of the inhibition of *Leishmania* viability for promastigote and amastigote-like of the three *Leishmania* species.

Leishmania species	IC_{50} (μg/mL)	
	Promastigotes	Amastigotes-like
L. amazonensis	67.5 ± 1.7	115.4 ± 2.1
L. chagasi	65.8 ± 1.7	112.3 ± 2.0
L. major	56.8 ± 1.7	108.4 ± 2.2

Tabela 2 - Inhibition of the infection in murine macrophages after treatment of promastigotes with the *Agaricus blazei* Murill water extract. The results are expressed as medium±standard deviation of the percentages of the infected macrophages by the non-treated and treated cultures of *L. amazonensis*, *L. chagasi*, and *L. major*.

Promastigotes	Percentage of infected macrophages by non-treated *Leishmania*	Percentage of infected macrophages by treated *Leishmania*	Infectiveness reduction
L. amazonensis	82.0 ± 2.7	12.7 ± 1.6	84.4 ± 2.3
L. chagasi	57.0 ± 1.3	24.5 ± 1.7	79.6 ± 1.9
L. major	73.0 ± 3.4	19.7 ± 1.4	85.3 ± 3.1

To provide additional information concerning the chemical entities in the *A. blazei* Murill water extract that can be responsible for the biological effects observed in this study, a chemical characterization was performed, as described in the Material and Methods section. The result of this characterization illustrated the presence of tannins, saponins, proteins, glycoproteins, polysaccharides, and carbohydrates within the water extract.

3.2. Inhibition of infection and treatment of infected macrophages

The *Leishmania* infectivity treated with the water extract was evaluated in murine macrophages. Parasites (*L. amazonensis*, *L. chagasi*, and *L. major*) that were not pre-incubated with the water extract were able to infect 82%, 57%, and 73% of the murine macrophages, respectively. When parasites were pre-incubated with 25 μg/mL of the water extract for 1 h, they were able to infect approximately 12.7%, 24.5%, and 19.7% of the murine macrophages, respectively, in turn presenting reductions in their infectivity in the order of 84.4%, 79.6%, and 85.3% for *L. amazonensis*, *L. chagasi*, and *L. major*, respectively. In this light, it can be deduced that pre-treatment with the water extract was able to inhibit the infection of *Leishmania* in macrophages (Table 2). An optical microscopy was carried out on macrophages infected with the untreated and treated parasites, and it could be observed that the parasites remained attached in the external membranes of phagocytic cells and were unable to be internalized within them, thus demonstrating a possible effect of the water extract in reducing the infection in the macrophages (Fig. 3).

In this manner, an evaluation of the capacity of the water extract in treating infected macrophages was also performed. Cells were first pre-infected with *L. amazonensis*, *L. chagasi*, or *L. major* promastigotes and later treated with 50 μg/mL of water extract for 4 h at 24°C. The subsequent results showed that murine macrophages that were infected and later

treated presented reductions in the parasite burden in the order of 44.1%, 30.1%, and 45.2% for *L. amazonensis, L. chagasi*, or *L. major*, respectively (Table 3). In addition, a confocal microscopy was performed, which indicated that, in the infected cells treated with the water extract, many intra-macrophage parasites, which were labeled with an orange color to reveal the presence of apoptotic DNA, could be observed when the three *Leishmania* species were tested. It is interesting to note that infected and untreated macrophages presented colonized vacuoles, demonstrating the importance of the treatment with the water extract in the infected cells (Fig. 4).

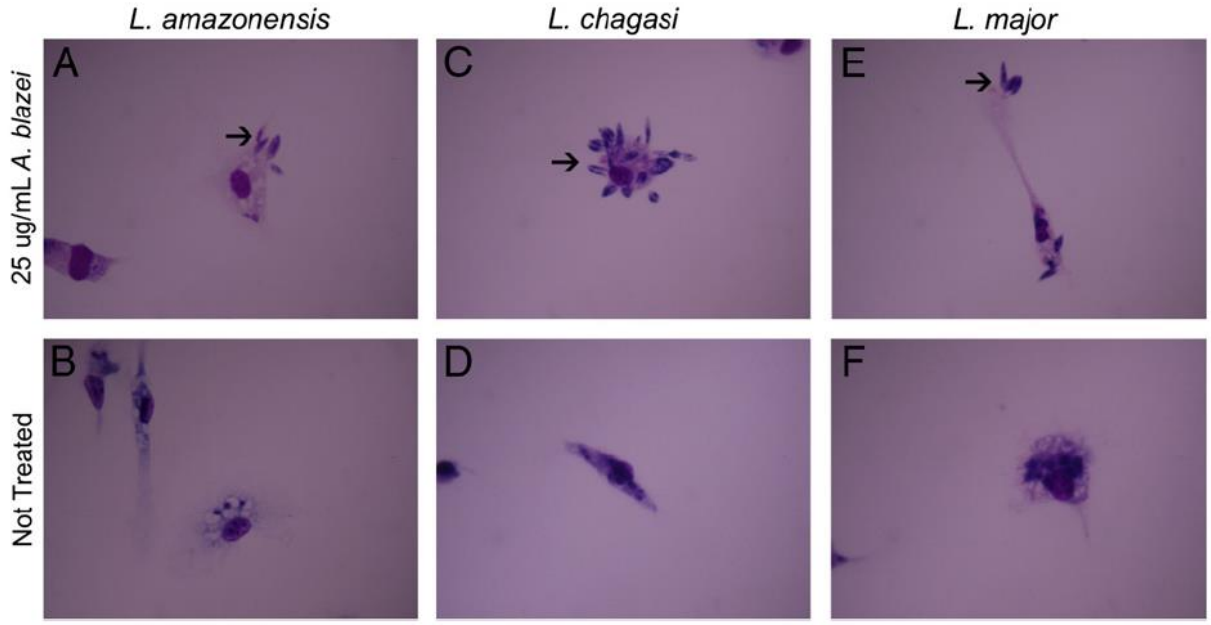

Figura 3-. Optical microscopy of the inhibition of the infection Leishmania in murine macrophages. Promastigotes were pre-incubated with the of *A. blazei* Murill water extract (25 μg/mL) for 1 h at 24 °C. After incubation, parasites were washed and used to infect macrophages (10:1 ratio) for 4 h at 24 °C. Optical microscopy of untreated Leishmania illustrates the presence of parasites within parasitophorous vacuoles in the infected macrophages (panels A, C, and E for *L. amazonensis, L. chagasi* and *L. major*, respectively). In panels B, D, and F, *L. amazonensis, L. chagasi*, and *L. major*, respectively, that had been pre-incubated with the water extract remained attached to the external membrane of the macrophages and were unable to infect the phagocytic cells.

Tabela 3- Percentage of infected macrophages and parasite burden after treatment with the *Agaricus blazei* Murill water extract. The results are expressed as medium±standard deviation of the percentages of the infected macrophages and the reduction of the parasite burden in treated cultures.

Promastigotes	Percentage of infected macrophages	Reduction of internalized parasites (%)
L. amazonensis	82.0 ± 2.7	44.1 ± 2.3
L. chagasi	57.0 ± 1.3	30.1 ± 1.8
L. major	73.0 ± 3.4	45.2 ± 3.1

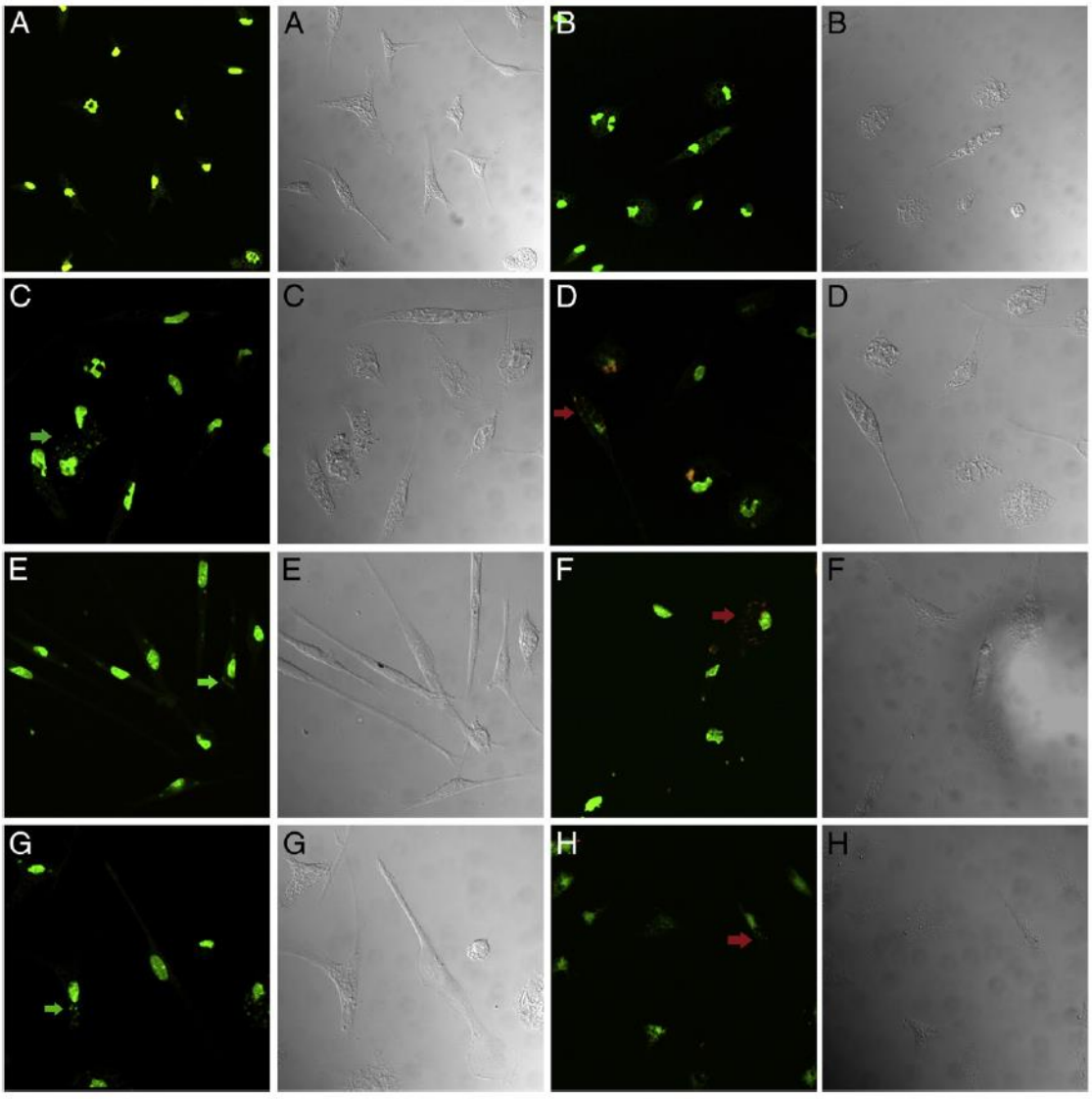

Figura 4- Confocal microscopy analysis of treatment of infected macrophages. Murine macrophages were infected with different *Leishmania* species (1:10 ratio, respectively) and later treated with the *A. blazei* Murill water extract (50 μg/mL). Panels A and B indicate uninfected and untreated macrophages, and uninfected macrophages that had been treated with the water extract, respectively. The labeling of the infected and untreated cells can be observed in panels C, E, and G for *L. amazonensis, L. chagasi, and L. major*, respectively (green arrows). In panels D, F, and H, macrophages infected with *L. amazonensis, L. chagasi*, and *L. major*, respectively, which were treated with the water extract. In these cases, there is a general overview of macrophages containing non-viable parasites (orange color).

3.3. Nitric oxide (NO) production and iNOS expression

The activation of macrophages by NO production after the treatment with the *A. blazei* Murill water extract was also investigated. The data showed no production of nitrite after 24, 48, and 72 h of incubation (Fig. 5). Concanavalin A (ConA, 5 μg/mL) was used as a positive control and demonstrated a high NO production. To confirm whether or not the water extract truly induced the NO production, the expression of iNOS was investigated by applying a Western-blot technique. As shown in Fig. 6, no iNOS expression could be detected in the macrophages that were incubated alone or stimulated with the water extract. However, after stimulation with ConA, cells were able to show an iNOS expression.

Figura 5- Nitric oxide (NO) production. Murine macrophages (4×105) were separately stimulated with the *A. blazei* Murill water extract (50 μg/mL) or Concanavalin A (5 μg/mL) for 24, 48, and 72 h, at 37 °C, in 5% CO2. Lines represent mean ± standard deviation of nitrite levels (μM) per group, after incubation at the different periods of time. Data shown are representative of three independent experiments, performed in triplicate, which presented similar results.

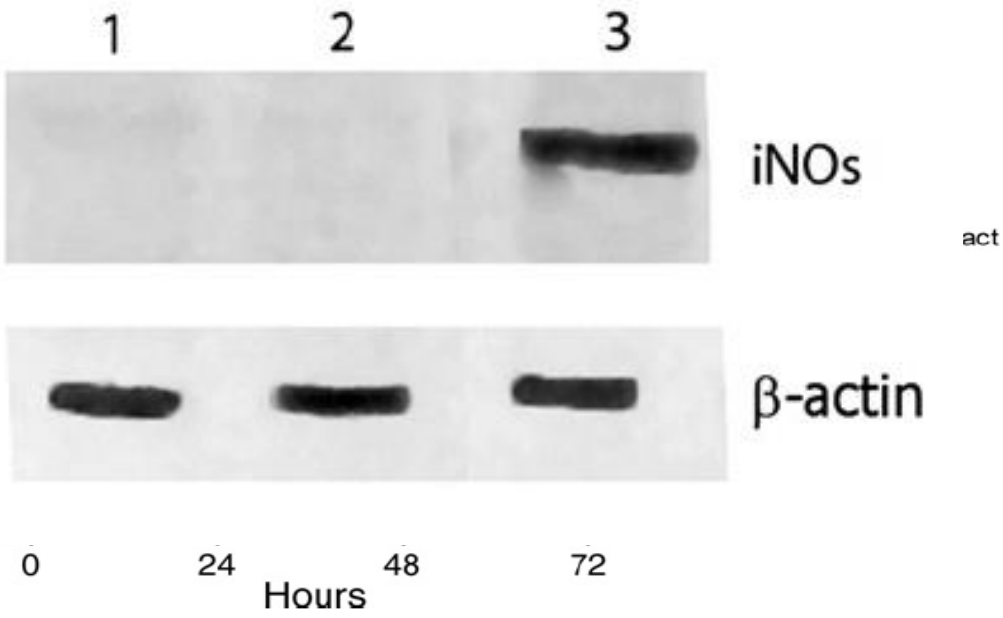

Figura 6 - Inducible nitric oxide syntase (iNOS) expression. Murine macrophages were incubated alone (medium; background control, in 1) or separately stimulated with the *A. blazei* Murill water extract (50 μg/mL, in 2) or Concanavalin A (5 μg/mL, in 3) for 24 h at 37 °C, in 5% CO2. Cells were lysed, and iNOS expression (123 kDa) levels were measured by Western-blot assay using specific antibodies. Beta-actin expression (45 kDa) was used as a standard.

3.4. Cytotoxicity and hemolytic activity

The cytotoxicity of the *A. blazei* Murill water extract was investigated in murine macrophages. The in vitro assay demonstrated no significant cytotoxicity in cells with a high concentration (200 μg/mL) of the water extract, especially when compared to amphotericin B, which, when used in the concentration of 1 μg/mL, was able to induce toxicity in 20% of the cells (Table 4). In addition, the hemolytic activity in O+ human red blood cells was also determined as a cytotoxic parameter, and no significant damage to human erythrocytes could be observed after incubation with 200 μg/mL of the water extract (Table 4).

Tabela 4- Cytotoxicity and hemolytic activity. The results are expressed as medium±standard deviation of the percentage of the cytotoxic and hemolytic activities of the *A. blazei* Murill water extract. ND: not done.

Treatment (μg/mL)	Cytotoxicity (%)	Hemolysis (%)
A. blazei extract (25)	3.15 ± 1.15	3.51 ± 1.04
A. blazei extract (50)	6.02 ± 0.89	2.73 ± 1.39
A. blazei extract (100)	12.31 ± 1.78	3.51 ± 1.17
A. blazei extract (200)	15.55 ± 1.98	4.32 ± 1.22
Amphotericin B (1)	20.00 ± 4.00	ND

4. DISCUSSION

Current treatment for visceral and cutaneous leishmaniasis is considered inadequate, given that drugs present high toxicity levels, high cost, a growing parasite resistance, and long-term treatments [23]. Compounds extracted from natural products represent an important source for research concerning new drugs to be used in developing novel therapeutic agents [24-30]. In this light, the purpose of this study was to evaluate the leishmanicidal activity of a water extract of the *Agaricus blazei* Murill mushroom against different *Leishmania* species, namely, *Leishmania amazonensis, L. major*, and *L. chagasi* promastigotes and amastigotes-like stages. The water extract showed a good leishmanicidal activity against the different *Leishmania* species and in both parasite stages. It proved to be effective in inhibiting the infection of phagocytic cells by parasites that had been pre-treated with the water extract, as well as in reducing the parasite burden in infected macrophages that were later treated with the extract. In addition, the water extract presented a low citotoxicity in murine macrophages and no hemolytic activity in human red blood cells. The biological activity of the mushroom can be considered relevant, as it was effective against *Leishmania* species found in different parts of the world. In this light, *L. major*, an important etiological agent of Old World Cutaneous Leishmaniasis, was the strain that proved to be most susceptible to the water extract, presenting IC_{50} values of 56.8 and 108.4 μg/mL for promastigote and amastigote-like stages, respectively. Similarly, the water extract also proved to be effective against *L. chagasi*, presenting IC_{50} values of 65.8 and 112.3 μg/mL, as well as against *L. amazonensis*, with IC_{50} values of 67.5 and 115.4 μg/mL for promastigote and amastigote-like stages, respectively. Results from the present study showed that the mushroom was more potent against *Leishmania* promastigotes than against amastigotes-like stages, since the IC_{50} values were more effective in inducing leishmanicidal activity in the amastigote-like stages. These differences can be, at least in part, explained by the distinct morphological and biochemical characteristics of both stages, given that the amastigotes can modulate the immune response of the hosts and, consequently, be more resistant than the promastigotes [31].

Macrophages represent the main host cells in leishmaniasis and play a relevant role in the immunological control of intracellular parasitism through the production of oxygen derivative metabolites [32]. Through the up-regulation of NO production within the cells, macrophages

can trigger an intracellular killing mechanism of the internalizated parasites [33]. Mushrooms are known for their immune properties that can be associated with the polysaccharide fractions. In particular, β-glucans are the main fractions responsible for these immune effects. Fungal β-glucans have been reported to activate leukocytes and can increase the phagocytic activity of the cells, the production of reactive oxygen intermediates, as well as the production of inflammatory mediators, such as cytokines and chemokines [14,34,35]. In a chemical characterization performed with the water extract used in this study, the presence of the tannins, saponins, proteins, glycoproteins, polysaccharides and carbohydrates could be observed. Therefore, the present study aimed to investigate the NO production and the iNOS expression by macrophages treated with an *A. blazei* Murill water extract. It could be observed that the extract did not induce NO production, nor iNOS expression, suggesting that the leishmanicidal activity observed may be due to a mechanism other than NO production by macrophages. Recently, Volman et al. (2010) reported that the *Agaricus bisporus* mushroom was able to induce a strong immune-stimulating effect on bone marrow-derived macrophages and NO production, whereas the *Agaricus blazei* Murill mushroom was unable to induce the NO production [36]. This result is in accordance with findings from the present study.

Tempone et al. (2001) showed that snake venom presented leishmanicidal activity without inducing NO production [37]. According to this data, together with that described in this work, it can be speculated that other mechanisms than NO production may well be involved in the elimination of parasites without requiring macrophage activation via iNOS expression. This finding suggests that the product can transpose the membrane of the macrophages and reach the internalized parasites through a direct effect of the water extract on the parasites. In this manner, a confocal microscopy revealed that infected macrophages that were later treated with the water extract, as compared to infected and untreated controls, presented important reductions in the parasite burden. An optical microscopy analysis performed in this work showed that parasites that were pre-incubated with the water extract, as compared to the controls, encountered several difficulties in infecting macrophages. As a low citotoxicity in the macrophages and a poor hemolytic activity in human red blood cells could be observed, it could be inferred that the water extract is safe for mammalian cells, considering that a high concentration of this product (up to 200 μg/ml) presented low citotoxicity in the hosts. Amphotericin B, when applied in a low doses (10 μg), proved to be quite efficient in

eliminating parasites *in vitro*, but it was highly cytotoxic in mammalian cells, thus demonstrating some of its reported adverse effects *in vivo*.

In conclusion, this research described the leishmanicidal effect of the *A. blazei* Murill water extract on different *Leishmania* species and in both stages. This study showed that the extract's activity was not mediated by NO production and that parasite elimination may well be due to a direct effect on the infected macrophages. Further studies may lead to the characterization of alternative molecular mechanisms and to their identification within isolated compounds of *A. blazei* Murill that can be purified and used in future studies on *in vivo* leishmanicidal activity. Thus, further exploration of this mushroom is recommended in an attempt to find suitable chemotherapeutic alternatives for the treatment of cutaneous and visceral leishmaniasis.

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CAPÍTULO 2

Therapeutic efficacy induced by the oral administration of *Agaricus blazei* Murill against *Leishmania amazonensis*. Parasitology Research, volume 111, número 4, p. 1807-1816, 2012.

2º objetivo da tese: Com o achado de que o extrato aquoso do fungo *Agaricus blazei* foi efetivo contra diferentes espécies de *Leishmania* nos experimentos *in vitro* realizados, o mesmo foi utilizado na formulação de preparações terapêuticas e avaliado no tratamento da infecção causada pela espécie *L. amazonensis* em camundongos BALB/c, no sentido de se verificar se as preparações seriam efetivos no tratamento da leishmaniose tegumentar murina. Uma série de experimentos foi conduzida e os resultados obtidos permitiram a publicação do artigo científico intitulado "Therapeutic efficacy induced by the oral administration of *Agaricus blazei* Murill against *Leishmania amazonensis*", que será apresentado a seguir, em sua íntegra.

Título do artigo:

Therapeutic efficacy induced by the oral administration of *Agaricus blazei* Murill against *Leishmania amazonensis.*

Referência no PUBMED:

Parasitology Research, volume 111, número 4, p. 1807-1816, 2012.

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Abstract

The development of therapeutic alternatives to treat Leishmaniasis has received considerable attention. The present study aimed to investigate the efficacy of the *Agaricus blazei* Murill water extract (AbM) to treat BALB/c mice infected with *Leishmania amazonensis*. First, a dose-titration curve was performed. The most well-defined concentration able to induce the most effective results in the infected animals, considering a daily administration of the product, was that of 100 mg/kg/day. In this context, the AbM was administered orally, beginning on day 0 up to 20 days post-infection. Additional animals were treated with amphotericin B (AmpB, 5 mg/kg/day) by peritoneal route for the same period of time, while the control group received distilled water. The animals were evaluated at 14 weeks post-infection, at which time the parasitological and immunological parameters were analyzed. Mice treated with the AbM presented a 60% reduction in the inflammation of infected footpads as compared to untreated control infected mice. Moreover, in the treated mice, as compared to the untreated controls, approximately 60% and 66% reductions could be observed in the parasite burdens of the footpad and draining lymph nodes, respectively. In addition, no parasites could be detected in the spleen of treated mice at week 14 post-infection. These treated animals produced significantly higher levels of IFN-γ and nitric oxide (NO), higher levels of parasite-specific IgG2a isotype antibodies, and lower levels of IL-4 and IL-10 in the spleen and lymph node cell cultures than did the controls. Differences could be observed by comparing animals treated with AbM to those treated with AmpB, as indicated by a significant reduction in tissue parasitism, higher levels of IFN-γ and NO, and lower levels of IL-4 and IL-10, as well as by a decreased hepatic toxicity. In conclusion, the present study's data show that the *A. blazei* Murill water extract presents a high potential for the treatment of leishmaniasis, although additional

studies on mice, as well as on other mammal hosts, are warranted in an attempt to determine this extract's true efficacy as compared to other known therapeutic products.

Keywords*:* *Leishmania amazonensis*; *Agaricus blazei* Murill; Mushroom; Oral Treatment, Tegumentary Leishmaniasis.

Introduction

Leishmaniasis is a group of vector-transmitted diseases that are endemic in 88 tropical and subtropical countries. Many geographic regions are endemic for multiple *Leishmania* species. This is the case in South America, where the disease is caused by at least eight different species of *Leishmania* (WHO 2000). American Tegumentary Leishmaniasis (ATL) includes a variety of forms that are commonly referred to by their clinical and pathologic features: Cutaneous Leishmaniasis (CL), Mucocutaneous Leishmaniasis (MCL), and Diffuse Cutaneous Leishmaniasis (DCL). *Leishmania amazonensis* is associated with all clinical forms of ATL (Afonso and Scott 1993; Grimaldi and Tesh 1993), as well as with Visceral Leishmaniasis (VL) in humans (Barral et al. 1991).

Historically, the treatment of leishmaniasis has been based on the use of pentavalent antimonials. The parenteral administration of these compounds is still the first choice therapy; however, increased parasite resistance and side effects, such as, arthralgias, myalgias, pancreatitis, leukopenia, and cardiotoxicity are important problems experienced by patients (Berman 2003; Croft and Coombs 2003; Oliveira et al. 2011). Liposomal amphotericin B (AmpB) is considered effective, though these formulations are very expensive (Mondal et al. 2010). Results from clinical trials of oral miltefosine treatment are encouraging; however, therapies using miltefosine are expensive, are linked to potential toxicity, and should not be given to pregnant or to childbearing age women (Kedzierski et al. 2009). Therefore, the development of alternative therapeutic strategies to treat leishmaniasis has become a high-priority (Frézard and Demicheli 2009). Over the past few decades, major emphasis has been given to the identification of new formulations for both oral and topical treatments of the disease (Berman 2005; Croft and Coombs 2003). In this context, new treatment routes, as

compared to parenteral administration, represent an interesting approach and offer several advantages, including improved safety, better compliance, and a lesser pain than that produced by needle-use administration (Aguiar et al. 2009; Croft and Olliaro 2011).

Agaricus blazei Murill is a commonly found mushroom in Brazil, and its use has been associated with folk medicine in the treatment of some diseases, like leukemia, cancer, and arterial hypertension (Kim et al. 2005; Talcott et al. 2007; Kim et al. 2009). Compounds such as β-D-glucans, glycoproteins, saponins, tannins, polysaccharides, steroids, ergosterol, and fatty acids have been detected in this mushroom, which have been shown to activate the host's immune response in different *in vitro* experiments (Sorimachi et al. 2001; Bernardshaw et al. 2005; Forland et al. 2010). Recently, the present study's research group showed that the *A. blazei* Murill water extract presents an effective *in vitro* antileishmanial activity against different *Leishmania* species, including *L. amazonensis* (Valadares et al. 2011). In addition to the elimination of parasites in the infected macrophages, the water extract showed no cytotoxic effects in either murine macrophages or human red blood cells.

The present study investigated the efficacy of the *A. blazei* Murill water extract in the treatment of BALB/c mice that had been experimentally infected by *L. amazonensis*, a highly susceptible mouse model. The extract was administered orally in BALB/c mice. Its capability of treating the infected animals was compared to that obtained through parenteral treatment using AmpB, by examining parasitological and immunological parameters.

Materials and Methods

Mice

The Committee on the Ethical Handling of Research Animals (CETEA) from Federal University of Minas Gerais (UFMG) approved all the animal handling methods and procedures (code 056/2010). Female BALB/c mice (8 weeks of age) were obtained from the breeding facilities of the Department of Biochemistry and Immunology, Institute of Biological Sciences, UFMG, and were maintained under specific pathogen-free conditions.

Parasites and antigen preparation

Leishmania amazonensis (IFLA/BR/1967/PH-8) was grown at 24ºC in Schneider's medium (Sigma, St. Louis, MO, USA), supplemented with 20% heat-inactivated fetal bovine serum (FBS, Sigma), 20 mM L-glutamine, 200 U/mL penicillin, and 100 µg/mL streptomycin, at pH 7.4. The soluble *L. amazonensis* antigenic extract (SLA) was prepared from $1x10^{10}$ stationary-phase promastigotes, as previously described by Coelho et al. (2003).

Agaricus blazei Murill water extract

The water extract was prepared by macerating 50 g of fresh mushrooms in 50 mL of sterile milli-Q water added to a protease inhibitor cocktail (Sigma, catalog P8340) using a Waring-Blendor homogenizer for 1 h at 4ºC. Next, the mixture was centrifuged at 10.000 x *g* for 30 min at 4°C (LC5C model, Sorval). The supernatant was then collected, sterilized, by being passing through a 0.22 µm membrane; and stored at -80ºC until use.

Infection and treatment regimens

BALB/c mice (n=12 per group) were infected in the right hind footpad with 2×10^5 stationary-phase promastigotes of *L. amazonensis*. Animals were divided into three groups, according to the regimens of treatment, each receiving the treatment once a day, beginning at day 0 of infection and followed up until 20 days post-infection: 1) treatment with the *A. blazei* Murill water extract (AbM, 100 mg/kg of body weight/day) by oral route, 2) treatment with Amphotericin B deoxycholate (AmpB, 5 mg/kg/day, Sigma, catalog A9528) by parenteral route, and 3) administration of distilled water by oral route. All animals were maintained in abstinence of food for 3 h pre-treatment and 1 h post-treatment.

Cutaneous lesion development

The course of the disease was monitored weekly by measuring the footpad thickness with an electronic caliper (799-6/150 model, Starrett®, Brazil), and expressed as the increase in thickness of the infected hind footpad, as compared to the uninfected left footpad. Mice were evaluated for lesion development for 14 weeks.

Estimation of parasite load

The mice (n=6 per group) were euthanized at 10 and 14 weeks post-infection, when the infected footpads, spleen, and draining lymph node (dLN) were collected for parasite quantitation by means of a limiting dilution assay, as described by Vieira et al. (1996). Briefly, the organs were weighed and homogenized, using a glass tissue grinder in sterile phosphate buffer saline (PBS). Tissue debris was removed by centrifugation at 150 × *g*, and cells were concentrated by centrifugation at 2000 × *g*. The pellet was resuspended in 1 mL of Schneider's insect medium supplemented with 20% FBS. Two hundred and twenty microliters of the resuspension were plated onto 96-well flat-bottom microtiter plates (Nunc, Nunclon®, Roskilde, Denmark) and diluted in log-fold serial dilutions in supplemented Schneider's culture medium with a 10^0 to 10^{-24} dilution. Each sample was plated in triplicate and read 7 to 10 days after the beginning of the culture at 24ºC. Pipette tips were discarded after each dilution to avoid carrying adhered parasites from one well to another. Results are expressed as the negative log of the titer (*i.e.*, the dilution corresponding to the last positive well) adjusted per microgram of tissue.

Cytokine analysis

To measure IFN-γ, IL-4, and IL-10, the spleen and dLN cultures (5 x 10^6 cells, each one) were collected at week 10 post-infection and stimulated with SLA *L. amazonensis* (50 μg/mL), in duplicate, on 24-well, flat-bottomed plates (Nunc) for 48 h at 37ºC, 5% CO_2. The IFN-γ, IL-4, and IL-10 levels were determined in the culture supernatants by commercial kits (cat. 555138, 555232, and 555252 to IFN-γ, IL-4, and IL-10, respectively; BD OptEIA™ Pharmingen), according to manufacturer's instructions.

Nitric oxide (NO) production

To view the macrophage activation via NO production, the spleen and dLN (5 x 10^6 cells, each) were stimulated with SLA *L. amazonensis* (50 μg/mL) for 48 h at 37^0C, 5% CO_2. Following incubation, 50 μL of culture supernatants were mixed with an equal volume of Griess reagent (Sigma). After an incubation of 30 min at room temperature, the nitrite concentration was calculated using a standard curve of known concentration.

Serum samples from the mice were collected to determine alanine transaminase (ALT) and aspartate transaminase (AST) levels. The dosages were performed using commercial kits (catalogs K049 and K048 for ALT and AST, respectively, Bioclin Quibasa Ltda., Brazil), according to manufacturer's instructions.

Analysis of humoral responses

SLA *L. amazonensis*-specific IgG, IgG1, and IgG2a antibodies were measured by ELISA (Coelho et al. 2003). Briefly, 96-well plates (Falcon model) were sensitized with SLA *L. amazonensis* (1 μg/well) for 18 h at 4ºC. Next, the plates were washed 5 times with PBS 1x/Tween 20 0.05%, and the wells were blocked with a PBS 1x/bovine serum albumin 10%/Tween 20 0.05% solution, for 2 h at 37ºC. The plates were then washed 7 more times under the same conditions, and serum samples (1:100 diluted) were added, in duplicate, for 1 h at 37ºC. After, the plates were washed 7 times; specific peroxidase-labeled antibodies for mouse IgG, IgG1, and IgG2a isotypes (Sigma) were added separately (1:5,000 diluted); and incubation occurred for 1 h at 37ºC. Next, the plates were again washed 7 times, at which time H_2O_2 and *o*-phenylenediamine were added for the development of reactions that occurred for 30 min in the dark and were stopped by the addition of 20 μL H_2O_2 2 N. Optical densities were read at 492 nanometers in an ELISA microplate spectrophotometer (LAB-660 model, LGC Biotechnology).

Statistical analysis

Statistical analysis was performed using the GraphPad Prism software (version 5.0 for Windows). The differences among the diverse groups were evaluated using the one-way ANOVA analysis, followed by Bonferroni's post-test for multiple comparisons. Differences were considered significant when $P<0.05$. Data shown are representative of two different experiments, which presented similar results.

RESULTS

In vivo efficacy of the *Agaricus blazei* Murill water extract to protect against *L. amazonensis*

In this study, an oral treatment employing an *A. blazei* Murill water extract (AbM) was applied in BALB/c mice experimentally infected with L. amazonensis. In previous in vivo experiments developed by the present study's group (data not shown), a dose–titration curve was performed to determine the best concentration of AbM able to induce the most effective results in treating the infected mice. It could be observed that the concentration of 100 mg kg−1 day−1, administered once a day up to 20 days postinfection, produced the most reliable results concerning the animal treatments. Therefore, in the present study, AbM was administered orally at this concentration from day 0 up to 20 days postinfection. As a result, a significant reduction in the average lesion sizes could be observed in the animals treated with either AbM or AmpB after the ninth week of infection, as compared to the control mice (Fig. 1). Animals from both treatment groups displayed similar results in the swelling of the footpad, while controls showed a significant increase in their infected foot−pads. The protective response was sustained up to 14 weeks postinfection, when the mice treated with AbM or AmpB were still able to control the increase in lesion size.

To evaluate the parasite burden in the animals, the infected footpads, spleen, and dLN were collected at weeks 10 and 14 postinfection. Both treatments, i.e. with AbM and AmpB, as compared to the control mice, resulted in signif- icant reductions in the parasite load, in nearly all evaluated organs, not including the dLN at week 14 postinfection, when results between the control and AmpB groups displayed no significant difference between them (Fig. 2). However,

animals treated with AbM, as compared to those treated with AmpB, displayed better results in reducing the parasite load, including a complete elimination of parasites in the spleen of the animals at week 10 postinfection, which was maintained until week 14 of the treatment follow-up.

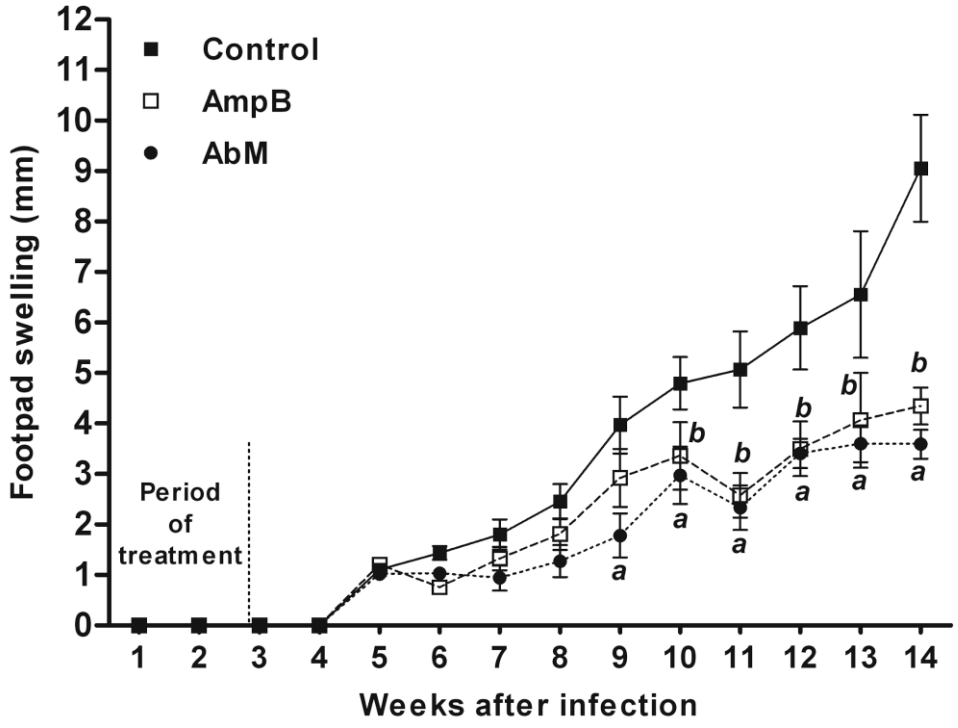

Figure 1-Footpad swelling in mice infected with *L. amazonensis* and treated with *A. blazei* Murill or amphotericin B. BALB/c mice (n=8 per group) were infected in the right hind footpad with 2 × 105 stationary-phase promastigotes of *L. amazonensis*. From day 0 of infection up to 20 days postinfection, animals were treated orally with the A. blazei Murill water extract (AbM, 100 mg kg−1 day−1), while another group received ampho- tericin B (AmpB, 5 mg kg−1 day−1) by parenteral route for the same period of time. An additional group received distilled water by oral route. All animals received treatments once a day. Lines indicate the mean±standard deviation (SD) of the lesion size (in millimeters) of the infected animals. Lesion development in the infected animals was monitored weekly up to week 14 postinfection. Significant differences

between the AbM and AmpB groups and the control group are indicated in the graph ("a" for AbM and control groups, and "b" for AmpB and control groups)

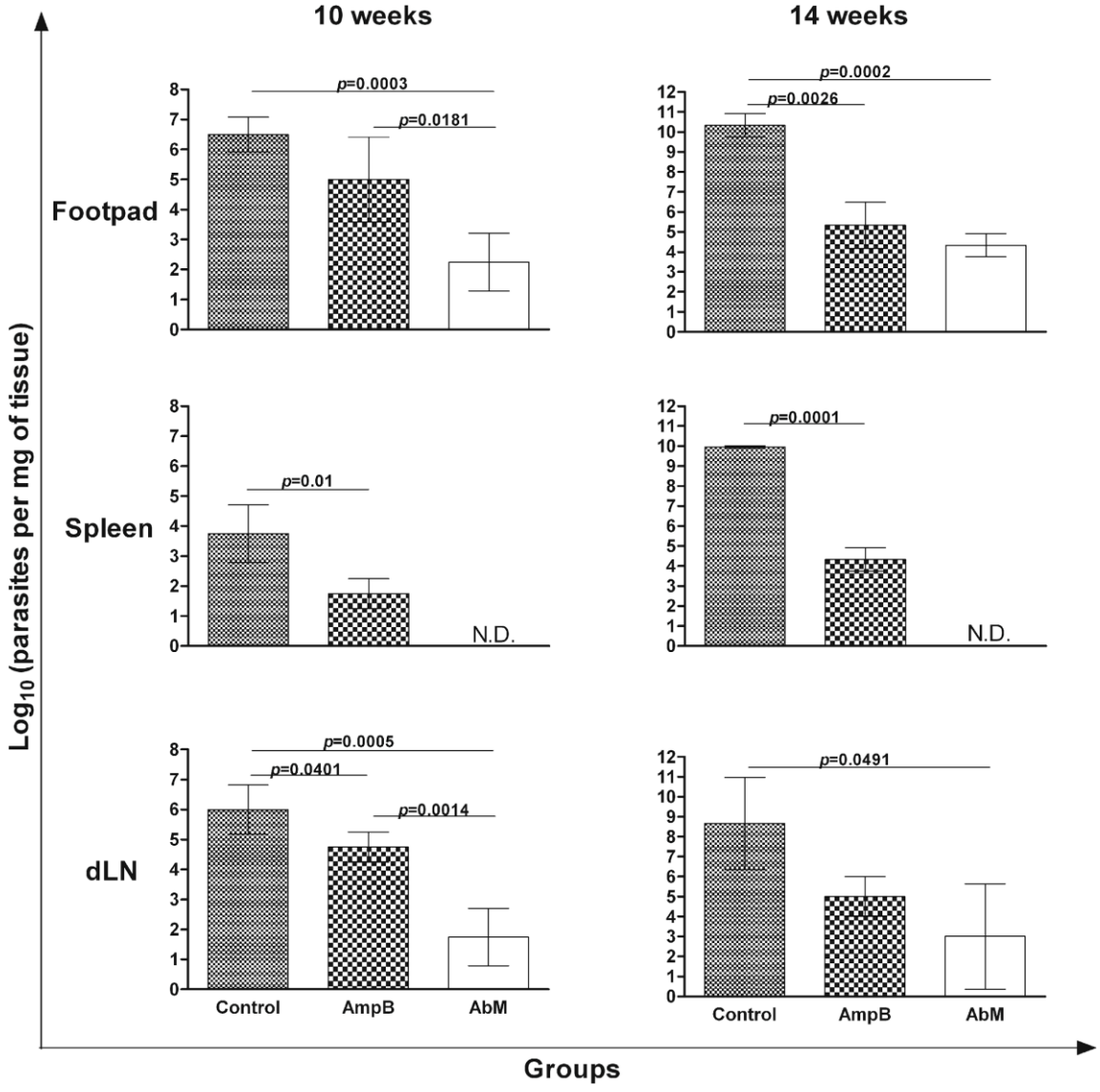

Figure 2- Parasite burden in the infected animals treated with *A. blazei* Murill or amphotericin B. BALB/c mice (n 0 8 per group) were infected in the right hind footpad with 2 × 105 stationary-phase promastigotes of *L. amazonensis* and were killed at weeks 10 and 14 postinfection. The parasite load was then determined in the infected footpads, spleen, and dLN. Bars indicate the mean ± standard deviation (SD) of the groups. Significant differences among the AbM, AmpB, and control groups are indicated in the graphs. N.D. Not detected

SLA-specific cellular and humoral responses elicited against *L. amazonensis*

To evaluate if the treatment altered immunological parameters associated with the resistance and/or susceptibility of BALB/c mice infected with *L. amazonensis*, the production of IFN-γ, IL-4, and IL-10 in the spleen and dLN culture supernatants was evaluated at week 10 postinfection. As shown in Fig. 3, spleen cells from mice treated with AbM produced significantly higher levels of SLA-specific IFN-γ than those secreted by spleen cells of animals treated with AmpB or control mice. Spleen cells were also incubated alone (nonstimulated, background control) and produced very low levels of all evaluated cytokines (data not shown). The SLA-specific IL-4 and IL-10 production was also investigated. Spleen cells from AbM-treated mice produced lower levels of IL-4 in relation to the levels detected in the control mice (Fig. 3). In the control group, in both splenic and dLN cultures, as compared to the IFN-γ levels, a higher production of IL-4 could be observed, suggesting a predom- inance not only in the draining site of infection but also in the systemic level of a Th2 response. In addition, the production of IL-10 by dLN in the AbM group proved to be significantly lower when comapred to the AmpB and control groups (Fig. 3).

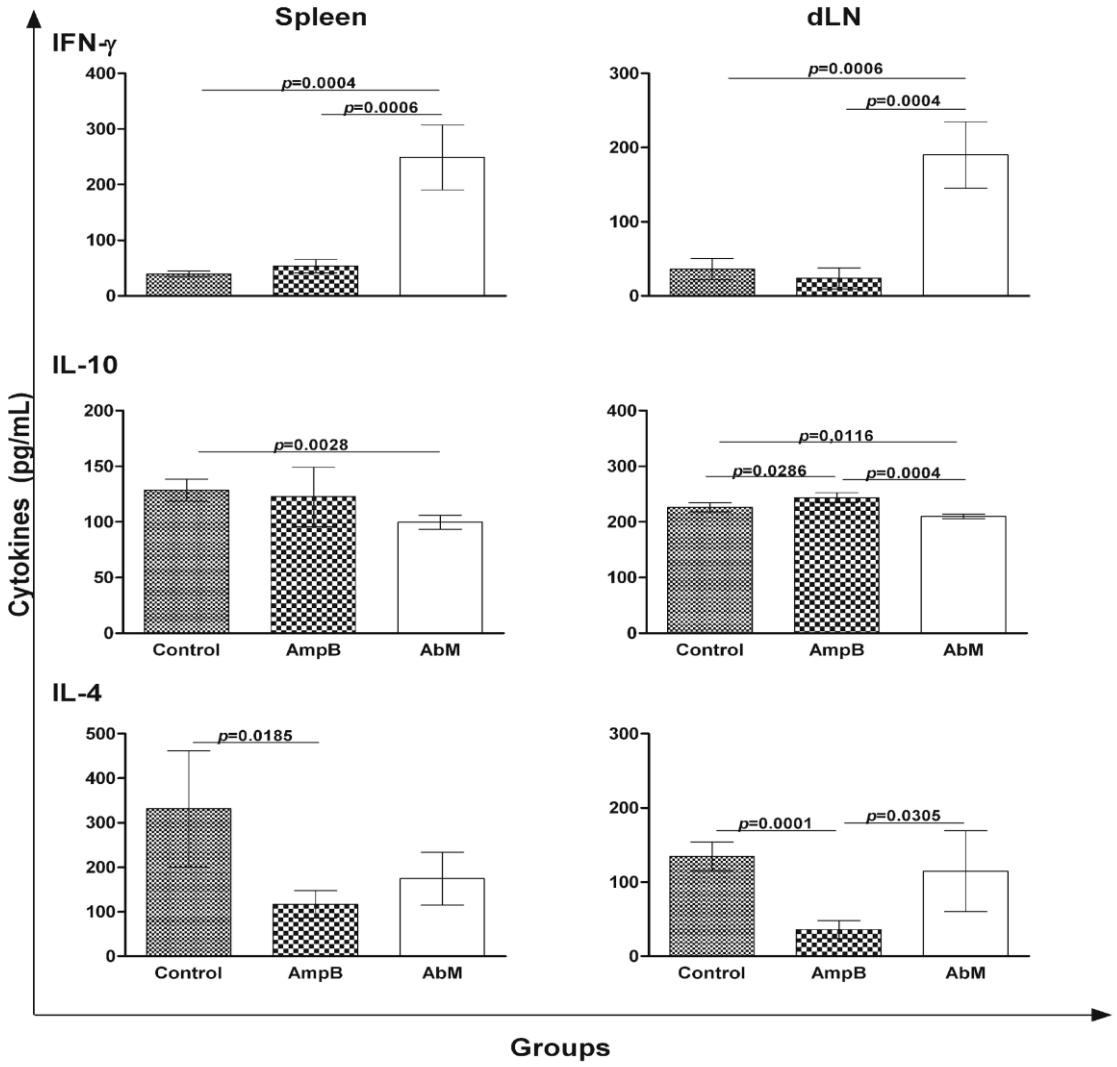

Figure 3- Cytokine levels in the infected animals treated with *A. blazei* Murill or amphotericin B. Single-cell suspensions were obtained from the spleen and dLN of infected and treated mice, 10 weeks post- infection. Cells were stimulated with SLA (50 µg/mL−1) for 48 h at 37 °C in 5 % CO2. IFN-γ, IL-4, and IL-10 levels were measured in culture supernatants by ELISA. Bars indicate the mean ± standard deviation (SD) of the groups. Significant differences among the AbM, AmpB, and control groups are indicated in the graphs.

The humoral response elicited against *L. amazonensis* in the groups was investigated in an attempt to determine the global anti-*Leishmania* antibody response induced after the treatments of the infected animals (Fig. 4). It could be observed that the control mice, as compared to the groups treated with AbM or AmpB, produced higher levels of SLA- specific IgG antibodies, as well as higher levels of IgG1 in relation to the IgG2a isotype. By contrast, treatment with AbM affected the global specific *L. amazonensis* humoral response, given that these animals produced lower levels of SLA-specific IgG antibodies and higher levels of IgG2a, as compared to the IgG1 isotype, suggesting the predominance of a Th1 immune response in the group that received the treatment with the *A. blazei* water extract.

To determine the influence of the treatments using AbM or AmpB as effector functions of infected macrophages in killing *L. amazonensis* killing, the nitrite production in the spleen and dLN of the animals was also assayed (Fig. 5). The nitrite production in spleen cell supernatants was sig- nificantly higher in the mice treated with AbM, as compared to those treated with AmpB or the control mice. Curiously, when dLN was used to determine the nitrite production, the levels obtained in the treated groups were lower than those obtained using splenic cell cultures.

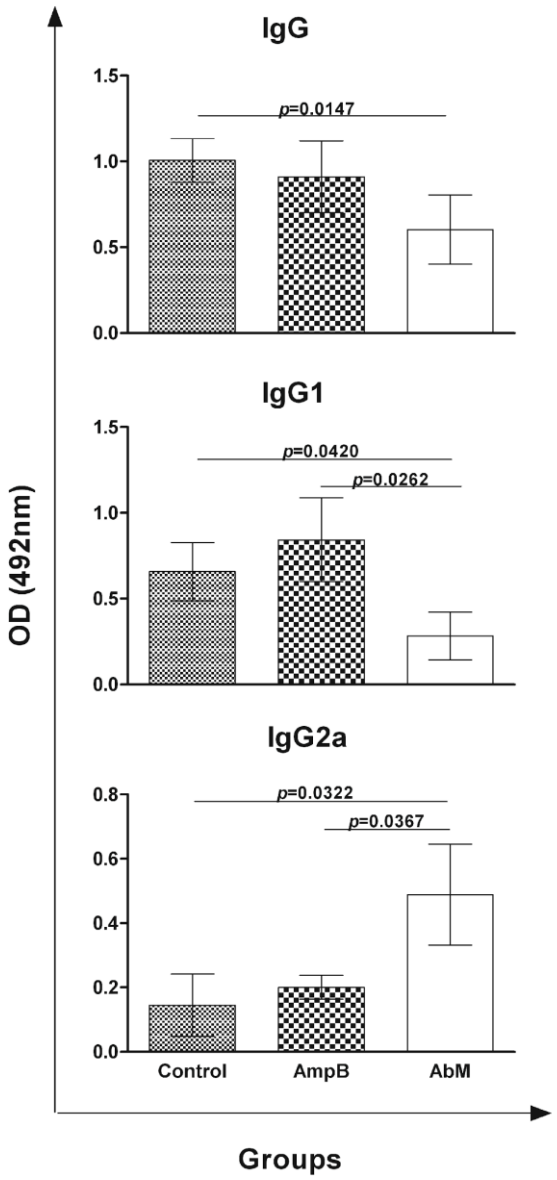

Figure .4 - SLA-specific antibody responses in the infected animals treated with *A. blazei* Murill or amphotericin B. Serum samples were obtained from the infected and treated mice, 10 weeks post-infection. Serum samples were tested by ELISA to determine the presence of SLA-specific IgG, IgG1, and IgG2a antibodies. Bars indicate the mean± standard deviation (SD) of the groups. Significant differences among the AbM, AmpB, and control groups are indicated in the graphs.

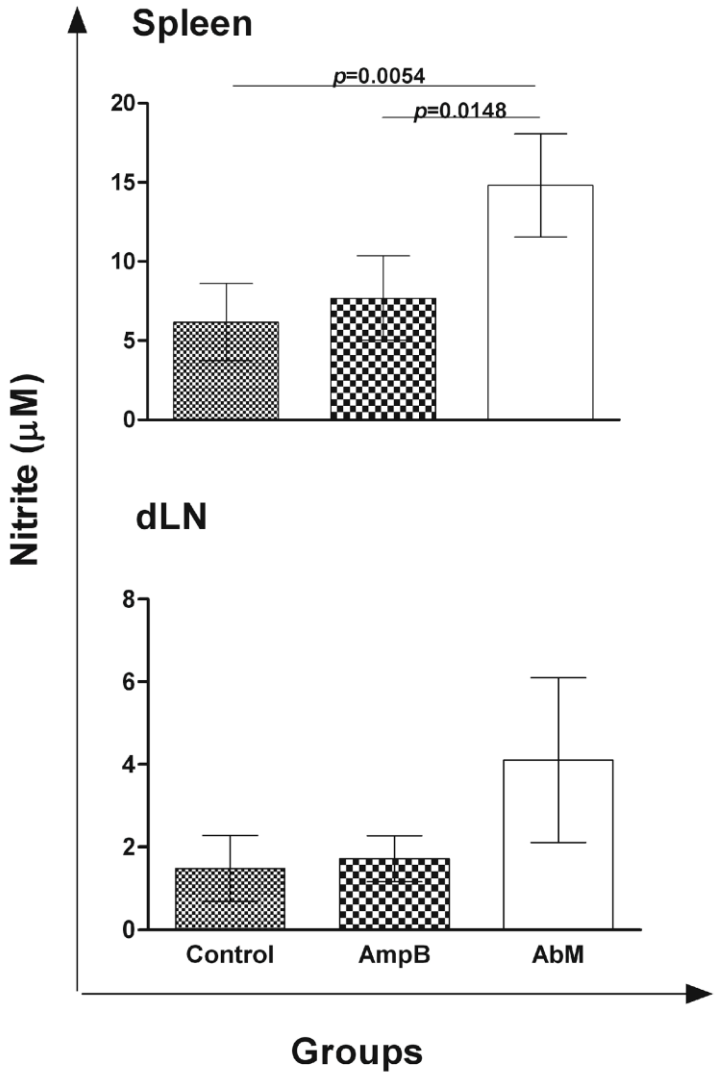

Figure 5- Nitric oxide production. Spleen and dLN cells (5 × 106) were stimulated with SLA (50 µg/mL−1) for 48 h at 37 °C in 5 % CO2. Following incubation, 50 µL of culture supernatants was mixed with an equal volume of Griess reagent (Sigma). After an incubation of 30 min at room temperature, nitrite levels (µM) were calculated using a standard curve of known concentrations. Bars indicate the mean± standard deviation (SD) of the groups. Significant differences among the AbM, AmpB, and control groups are indicated in the graphs.

The hepatic toxicity of the treatments using AbM or AmpB was also investigated in the animals. Serum samples of the mice were collected and the ALT and AST levels were determined using commercial kits. The data presented in Fig. 6 show that mice treated with AmpB displayed higher levels of ALT and AST when compared to the levels obtained in the animals treated with AbM. Moreover, the levels of ALT and AST in animals treated with AbM were comparable to those detected in noninfected/nontreated mice, suggesting the absence of hepatic toxicity in the AbM-treated mice.

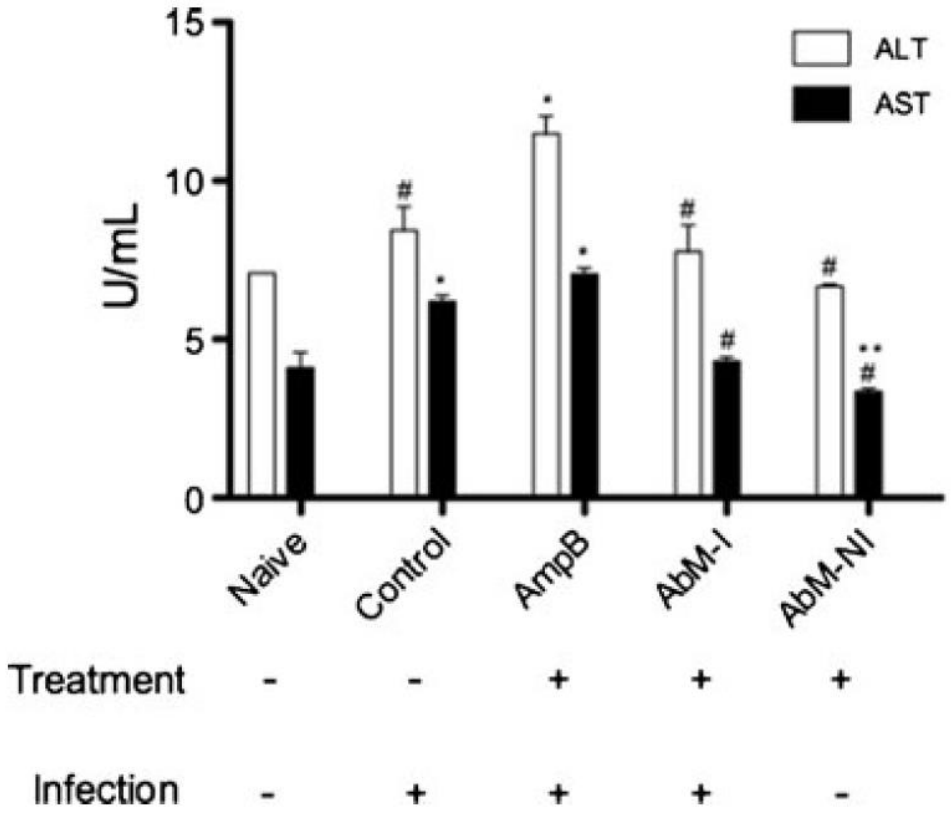

Figure 6- Serum levels of alanine transaminase and aspartate transaminase. Serum samples of naive, control (distilled water), AmpB, or AbM-treated and infected (AbM-I), or AbM-treated (AbM-NI) and non-infected mice were collected to determine the serum levels of alanine transaminase (ALT) and aspartate transaminase (AST) using commercial kits. White (ALT) and black (AST) bars indicate the mean ±standard deviation (SD) of the groups. The single asterisk indicates significant differences in relation to the naive mice; double asterisk indicates significant differences in relation to the control mice, and the number symbol indicates significant differences in relation to the AmpB group.

DISCUSSION

The development of new therapeutic strategies and products to treat leishmaniasis has received considerable attention in recent years, including the use of new formulations administered by oral and topical routes (Aguiar et al. 2009, 2010; Croft and Olliaro 2011). The purpose of this study was to analyze whether or not the *A. blazei* Murill water extract was effective against different *Leishmania* species *in vitro* experiments (Valadares et al. 2011) when administered at day 0 of infection and continuing up to 20 days postinfection, as well as whether or not it was capable of treating BALB/c mice experimentally infected with *L. amazonensis*.

The experimental model used in this study is extremely susceptible and can be considered a highly stringent model for experimental chemotherapy, given that BALB/c mice infected with *L. amazonensis* commonly develop a progressive lesion at the inoculation site, followed by ulceration and loss of tissue, which occurs simultaneously with the appearance of visceralization and metastasis at distant sites (Cupolilo et al. 2003). This model is generally refractory to treatment, especially when treatment is administered to developed lesions that have been followed up to check for progressive infections. In this case, the treatments do not commonly lead to significant reductions in parasitism and/or cures, unless additional interventions, such as the association of drugs, are employed (Aguiar et al. 2010). Therefore, in the present study, the treatment with AbM began immediately after infection, and its effect was directly compared to AmpB treatment, a well-known model of treatment for leishmaniasis, before the parasite burden had reached increased levels in the infected animals. In this context, both AbM and AmpB treatments were able to promote a significant reduction in both lesion size and tissue parasitism in the treated and infected animals. However, at approximately 10 weeks postinfection, the AbM treatment, as compared to AmpB therapy, proved to be more effective, leading to a significant reduction in the parasite burden in different sites, including a complete elimination of the parasites in the spleen of the treated and infected animals, which could be observed at 10 and 14 weeks postinfection. This is consistent with previous reports indicating that the *A. blazei* Murill water extract presents an effective *in vitro* anti-leishmanial activity against different *Leishmania* species, including *L. amazonensis* (Valadares et al. 2011). It was also possible to verify that the treatment with AbM was effective in inducing an elevated Th1 immune response in the infected animals. These mice presented a higher IFN-γ and NO production, associated with the predominance of *L. amazonensis*-specific IgG2a isotype

antibodies. By contrast, control BALB/c mice that had been chronically infected with *L. amazonensis* displayed high levels of IL-4 or IL-10 by splenic and dLN cell cultures, and a higher production of IgG1 isotype antibodies. These results are in agreement with Padigel et al. (2003), who showed that low levels of IL-10 produced by splenic cultures of BALB/c mice infected with *L. amazonensis* can favor a higher production of IFN-γ and NO and that low levels of IL-4 are also important to a healing phenotype in the infected animals with *L. amazonensis.*

Macrophages represent the main infected host cells in leishmaniasis and play a relevant role in the immunological control of intracellular parasitism, through the production of oxygen derivative metabolites (Balaraman et al. 2004). Through the upregulation of NO production within the cells, macrophages can trigger intracellular killing mechanisms of the internalized parasites (Van Assche et al. 2011). Therefore, leishmaniasis treatment efficacy is dependent on synergic interactions between the anti-Leishmania effect of drugs and the immune responses of the animals, in turn leading to the production of IFN-γ and NO. The present study observed that mice treated with AbM produced high levels of NO. In this context, the increased ability of AbM-treated *L. amazonensis* infected macrophages to produce NO may be associated with the decreased parasite loads observed in these mice. It is also possible that the higher production of IFN-γ and lower levels of IL-4 and IL-10 observed in these animals have limited the multiplication of parasites, thus leading to a reduction in the tissue parasitism and, consequently, for the activation of parasite killing mechanisms of the macrophages. In this context, Sorimachi et al. (2001) reported that the *A. blazei* mushroom is able to induce a strong immune-stimulating effect based on the NO production in bone marrow-derived macrophages. Bernardshaw et al. (2006) also showed that the AbM is able to induce the of NO and proinflammatory cytokine (IL-1, IL-6, IL-8, and tumor necrosis factor alpha) production, but not of the IL-10, in human monocytes. Similarly, Tang et al. (2009) showed that this mushroom is also effective in inducing the production of high levels of IFN-γ and low levels of IL-4 and IL-10 in spleen cell supernatants of naive BALB/c mice. Altogether, these data indicate that treatment with AbM induced infected BALB/c mice toward a *L. amazonensis*-specific Th1 immune response that was maintained during infection, allowing these animals to prevent the development of a more severe disease, as was also observed in the control mice.

Prior reports have associated mushroom immune properties with polysaccharide fractions. Similarly, β-glucans fungi have also been reported to activate leukocytes and to increase the phagocytic activity of these cells, monocytes, and granulocytes; the production of reactive oxygen intermediates; as well as the production of inflammatory mediators, such as cytokines and chemokines (Sorimachi et al. 2001; Bernardshaw et al. 2006, 2007). A chemical characterization was performed to provide additional information concerning the chemical entities in the *A. blazei* Murill water extract that may well be responsible for the antileishmanial biological effects observed in the present study. The result illustrated the presence of tannins, saponins, glycoproteins, and polysaccharides within AbM.

Finally, no hepatic toxic effect could be observed in the AbM-treated mice. By contrast, in the animals treated with AmpB, significant elevations in the ALT and AST levels could be detected, indicating a possible hepatic damage caused by the use of AmpB in the animals, as previously described by Croft and Coombs (2003). In this context, the present study's results are in agreement with findings from Hsu et al. (2008) and Wu et al. (2011), whose studies showed that *in vivo* treatment with *A. blazei* led to the reduction of ALT and AST levels in rats in models of chronic hepatitis B and hepatic fibrosis, respectively, as well as induced a healing phenotype in the treated and infected animals.

In conclusion, this study's data show that the *A. blazei* Murill water extract presents a high potential to be employed in the treatment of leishmaniasis, although additional studies are necessary to determine its efficacy in association with well-known therapeutic products, as well as its efficacy in other mammal models.

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CAPÍTULO 3

Prophylactic or therapeutic administration of *Agaricus blazei* Murill is effective in treatment of murine visceral leishmaniasis. *Experimental Parasitology,* volume 132, número 2, p. 228–236, 2012.

3º objetivo da tese: Os resultados obtidos utilizando o extrato de *Agaricus blazei* no tratamento da doença causada por *L. amazonensis* estimularam a realização de um fracionamento desse extrato no sentido de que as frações obtidas, mais purificadas, após os testes experimentais *in vitro* de verificação de suas atividades leishmanicidas realizados, fossem empregadas em experimentos i*n vivo.* Das frações obtidas, a Fa.b5 foi a que apresentou os melhores resultados e, dessa forma, foi empregada nos estudos *in vivo*. Juntamente com o extrato aquoso, os produtos foram utilizados no tratamento e prevenção (quimioprofilaxia) da infecção causada pela espécie *L. chagasi* em camundongos BALB/c. Os resultados obtidos permitiram a publicação do artigo científico intitulado "Prophylactic or therapeutic administration of *Agaricus blazei* Murill is effective in treatment of murine visceral leishmaniasis", que será apresentado a seguir, em sua íntegra.

Título do artigo:

Prophylactic or therapeutic administration of *Agaricus blazei* Murill is effective in treatment of murine visceral leishmaniasis.

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ABSTRACT

The present study aimed to investigate the *in vitro* antileishmanial activity of five fractions obtained from *Agaricus blazei* water extract (AbM), namely, Fab1, Fab2, Fab3, Fab4, and Fab5; and use the selected leishmanicidal fraction to treat BALB/c mice infected with *L. chagasi*. A curve dose-titration was performed to obtain the concentration to be test in infected animals. In this context, Fab5 fraction and AbM were used in the doses of 20 and 100 mg/kg/day, respectively, with the product been administered once a day. The effect induced by a chemo-prophylactic regimen, based on the administration Fab5 fraction and AbM 5 days before infection, and maintained for an additional 20 days post-infection was compared to a therapeutic regimen, in which the compounds were administered from 0 to 20 days of infection. Control animals were either treated with amphotericin B deoxycholate (AmpB) or received distilled water. All groups were followed up for 10 weeks post-infection, when parasitological and immunological parameters were analyzed. The Fab5 presented the best results of *in vitro* leishmanicidal activity. In the *in vivo* experiments, the use of Fab5 or AbM, as compared to control groups, resulted in significant reduced parasite burdens in the liver, spleen, and draining

lymph nodes of the infected animals, as compared to control groups. A Type 1 immune response was observed in the Fab5 or AbM treated animals. No significant toxicity was observed. The chemo-prophylactic regimen proved to be more effective to induce theses responses. In this context, the data presented in this study showed the potential of the purified Fab5 fraction of AbM as a therapeutic alternative to treat VL. In addition, it can be postulated that this fraction can be also employed in a chemo-prophylactic regimen associated or not with other therapeutic products.

Keywords: Visceral leishmaniasis, *Agaricus blazei* Murill, purified fractions, chemo-prophylaxis, oral treatment, BALB/c mice.

1. Introduction

Leishmaniasis is a group of vector-transmitted diseases that are endemic in 88 tropical and subtropical countries. Many geographic regions are endemic for multiple *Leishmania* species. This is the case in South America, where the disease is caused by at least eight different species of *Leishmania* (WHO, 2009). American Tegumentary Leishmaniasis (ATL) includes some forms that commonly refer to their clinical and pathologic features: Cutaneous Leishmaniasis (CL), Mucocutaneous Leishmaniasis (MCL), and Diffuse Cutaneous Leishmaniasis (DCL) (Grimaldi and Tesh, 1993). By contrast, human and canine Visceral Leishmaniasis (VL) in South America is mostly related to infections with *Leishmania chagasi* (Maia and Campino, 2008).

The current treatment for leishmaniasis has been based on the use of pentavalent antimonials. The parenteral administration of these compounds is still the first choice for therapy; however, increased parasite resistance and several side effects are important problems reported by patients (Croft and Coombs, 2003; Minodier and Parola, 2007). Liposomal amphotericin B (AmpB) is effective, but highly cost for the majority of patients (Mondal et al., 2010). Results from clinical trials of oral miltefosine treatment are encouraging; however, therapy is linked to potential toxicity, resistance, and teratogenicity, and should not be given to pregnant or to childbearing age women (Oliveira et al., 2011a). Therefore, the development of new therapeutic strategies to treat leishmaniasis has become a high-priority (Frézard and Demicheli, 2009). In this context, major emphases have been given to the identification of new and lower toxic compounds and alternative administration routes (Garnier and Croft, 2002; Berman, 2005; Giudice and Campbell, 2006; Aguiar et al., 2010; Carneiro et al., 2010).

Agaricus blazei Murill is a commonly found mushroom in Brazil, and its use has been associated with folk medicine in the treatment of some diseases, like leukemia, cancer, and arterial hypertension (Kim et al., 2005; Talcott et al., 2007; Kim et al., 2009). Compounds, such as β-D-glucans, glycoproteins, saponins, tannins, cerebrosides, polysaccharides, steroids, such as ergosterol, and fatty acids have been detected in this mushroom, which have been shown to activate and/or modulate the host immune response (Sorimachi et al., 2001; Bernardshaw et al., 2005; Forland et al., 2010). Recently, ~~a~~n *in vitro* antileishmanial activity against *L. amazonensis*, *L. chagasi*, and *L. major* was demonstrated for an *A. blazei* water extract (Valadares et al., 2011).

The present study investigated the antileishmanial activity of five purified fractions of the *A. blazei* water extract, namely, Fab1, Fab2, Fab3, Fab4, and Fab5. For those fractions presenting the best anti-*L chagasi* activity *in vitro L. chagasi* promastigotes growth inhibition assays (Fab4 and Fab5), as well as for the *A. blazei* water extract (AbM), the leishmanicidal effects on intra-macrophage *Leishmania*, as well as cytotoxic effects on murine macrophages and human red blood cells, were studied. Afterwards, the Fab5 and AbM were tested *in vivo* using the experimental infection of BALB/c mice with *L. chagasi* as the model, through comparison of chemo-prophylactic and therapeutic regimens. Their effects were also compared with the parenteral administration of AmpB by examining parasitological and immunological parameters. Besides, a phytochemical screening was carried out in the AbM and Fab5, in order to indicate the substances involved in the therapeutic efficacy of the both products.

2. Material and Methods

2.1. Mice and parasites

The Committee on the Ethical Handling of Research Animals (CETEA) from the Federal University of Minas Gerais (UFMG) approved all animal handling methods and procedures (code 056/2010). Female BALB/c mice (8 weeks of age) were obtained from the breeding facilities of the Department of Biochemistry and Immunology, Institute of Biological Sciences, UFMG, and were maintained under specific pathogen-free conditions. *Leishmania chagasi* (MHOM/BR/1970/BH46) was grown at 24ºC in Schneider's medium (Sigma, St. Louis, MO, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma), 20 mM L-glutamine, 200 U/mL penicillin, and 100 µg/mL streptomycin, at pH 7.4. The soluble *L. chagasi* antigenic extract (SLA) was prepared from a 1 x 10^{10} stationary-phase of promastigotes, as described (Coelho et al., 2003).

2.2. Preparation of the *Agaricus blazei* water extract and purified fractions

For AbM water extract preparation, 28 g of the fresh mushroom was macerated in 50 mL of milli-Q water containing a protease inhibitor cocktail (Sigma, code P8340). The product was macerated in an ice bath and maintained for 1 h at 4ºC, at which time it was filtered on paper filter to remove the insoluble particles. Lately, the AbM was submitted to centrifugations at

8.000 x *g* for 45 min at 4ºC, using different Amicon columns with different molecular weight cut-off (daltons – Da). Thus, Fab5 (molecules >100,000 Da), Fab4 (between 100,000 and 50,000 Da), Fab3 (between 50,000 and 10,000 Da), Fab2 between 10,000 and 3,000 Da), and Fab1 (< 3,000 Da) were selected. The AbM and the purified fractions were lyophilized and maintained at -80ºC, until use.

2.3. Chemicals

Acetonitrile and methanol (HPLC grade) were purchased from Tedia (Fairfield, OH, USA) and J.T. Baker (Phillipsburg, NJ, USA), respectively. Concentrated phosphoric acid (85% w/v, Merck, Darmstadt, Germany) was used. Ultrapure water was obtained using a Milli-Q plus system (Millipore, Milford, MA, USA). HPLC grade reference substances used were gallic acid (GA, 98%); epigallocatechin (EGC, 90%), catechin (C, min. 98%), epigallocatechin (GC, 98%), epicatechin (EC, 90%); epigallocatechin gallate (EGCG, 95%), purchased from Sigma (Milwaukee, WI, USA).

2.4. Phytochemical analysis and HPLC characterization of AbM and Fab5

The presence of tannins, flavonoids, coumarins, quinones, alkaloids, triterpenes and steroids and saponins was evaluated in AbM and Fab5 by thin layer chromatography (TLC) analysis and specific reagents (Wagner et al., 1984). Analyses were carried out on the Waters 2995 system (USA) composed of quaternary pump, auto sampler (model 2695), photodiode array detector (model 2996) and Empower software for data processing. An ODS C-18 LiChrospher® (125 x 4.0 mm I.D., 5µm; Merck) was employed at a temperature of 30ºC and flow rate of 1.0 mL/min. UV-photodiode array detection was performed at λ 210 nm. UV spectra from λ 210 to 400 nm were recorded on line for peak identification. A linear gradient of aqueous 0.1% phosphoric acid (A) and acetonitrile (B) was employed: A-B (95:5, v/v) to A-B (5:95, v/v), in 60 min, followed by 5 min of linear gradient 95% de A and 5% B. The AbM, Fab5 and reference compounds (GA, EGC, C, GC, EGCG) were dissolved in methanol to concentrations of 10, 5 and 1 mg/mL, respectively (Tarascou et al., 2010). After centrifugation at 8400 x *g* for 10 min, a volumn of 10 µL of the sample solutions were automatically injected onto the apparatus. The presence of GA in AbM and Fab5 was also confirmed for co-injection experiments. Proteins were detected in SDS-PAGE 10% gels by

silver staining, while glycoproteins presence were demonstrated in SDS-PAGE 10% stained by periodic acid Schiff. The presence of carbohydrates was investigated using a phenol–sulfuric acid method (Dubois et al., 1956).

2.5. Evaluation of the *in vitro* antileishmanial effects of the *A. blazei* preparations

The activity of the purified fractions of *A. blazei* was assessed *in vitro* by cultivating promastigotes of *L. chagasi* (4×10^5 cells) in the presence of different individual concentrations (10 to 200 µg/mL) of Fab1, Fab2, Fab3, Fab4, and Fab5 on 96-well culture plates (Corning Life Sciences, Corning, NY, USA) for 48 h at 24ºC. Cell viability was assessed by measuring the cleavage of 2 mg/mL of MTT [3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide] (Sigma). Absorbance levels were measured by using a multi-well scanning spectrophotometer (Labtrade, model 660) at a wavelength of 570 nm. As controls, AbM (10 to 200 µg/mL) and AmpB (0.1 to 10 µg/mL, Sigma, code A9528) were assayed. All analyses were performed in triplicate, and the product concentrations needed to inhibit 50% of the parasites´ viability (IC_{50}) were calculated by applying a sigmoidal regression of the concentration-inhibition curves.

2.6. Citotoxicity and hemolytic activity

Citotoxicity was evaluated by an MTT assay by cultivating macrophages (4×10^5) in different concentrations (10 to 200 µg/mL) of Fab4, Fab5, or AbM on 96-well plates for 24 h. Analyses were performed in triplicate, and the product concentrations needed to inhibit 50% of the macrophage viability (CC_{50}) was calculated by applying a sigmoidal regression of a concentration-inhibition curve. As control, AmpB (0.1 to 10 µg/mL) was assayed. In addition, the hemolytic activity was evaluated by incubating the Fab4, Fab5, or AbM at different individual concentrations (10 to 200 µg/mL), using a 5% red blood cell (human O^+) suspension for 1 h at 37ºC, in which the erythrocyte suspension was centrifuged (1000 x *g* for 10 min), and cell lysis was determined spectrophotometrically (540 nm). Analyses were also performed in triplicate, and the product concentrations needed to produce the lysis of 50% of the red blood cells (RBC_{50}) was calculated by applying a sigmoidal regression of a concentration-inhibition curve.

2.7. Treatment of infected macrophages

Murine peritoneal macrophages were plated on round glass coverslips within the wells of a 24-well culture plate (Nunc, Nunclon®, Roskilde, Denmark) at a concentration of 5 x 10^5 cells per coverslip in RPMI 1640 medium supplemented with 20% FBS, 2 mM L-glutamine, 200 U/mL penicillin, and 100 µg/mL streptomycin, at pH 7.4. After 2 h of incubation at 37°C in 5% CO_2, stationary-phase promastigotes of *L. chagasi* were added to the wells (5 x 10^6), and the cultures were incubated for 4 h at 37°C, 5% CO_2. Next, free parasites were removed by extensive washing with an RPMI 1640 medium, and infected macrophages either remained non-treated or were treated for 48 h with the Fab4, Fab5, AbM or AmpB (10, 10, 50 and 1 µg/mL, respectively). Cells were washed in RPMI 1640, incubated with 4% paraformaldehyde for 15 min, treated with 70% ethanol in an ice-bath for 4 h and, finally, washed three times with sterile PBS. The percentage of infected cells and the number of amastigotes per macrophage were analyzed by counting 200 cells, in triplicate, and treated and non-treated macrophages were compared.

2.8. *In vivo* regimens

Different mice groups were employed (n=12, per group). Two groups received the Fab5 fraction or the AbM (20 and 100 mg/kg/day, respectively), 5 days before infection by oral route. Two other independent mice groups received the same treatment, at 0-day of infection. For all four groups, treatment was continued for up to 20 days post-infection. Control groups were treated with amphotericin B deoxycholate (AmpB, 5 mg/kg/day, Sigma, code A9528) from days 0 to 20 post-infection by parenteral route or received distilled water. Additional animals received an oral administration schedule with Fab5 or AbM for 20 days and were not infected. All groups were treated once a day.

2.9. Infection

Mice were infected in their right hind footpad with 1 x 10^7 stationary-phase promastigotes of *L. chagasi.* All animals received follow-up for 10 weeks, at which time they were euthanized, and the liver, spleen, and draining lymph nodes (dLN) were collected to determine parasite burden and cytokine evaluation in the same period of time.

2.10. Estimation of parasite load

The spleen, liver, and dLN of the infected mice were used for parasite quantitation, following a technical protocol previously described (Vieira et al., 1996). Briefly, organs were weighted and homogenized, using a glass tissue grinder in sterile PBS. Tissue debris was removed by centrifugation at 150 × *g*, and cells were concentrated by centrifugation at 2000 × *g*. The pellet was resuspended in 1 mL of Schneider's medium supplemented with 20% FBS. Two hundred and twenty microliters of the suspension were plated onto 96-well flat-bottom microtiter plates (Nunc) and diluted in log-fold serial dilutions in supplemented Schneider's culture medium at a 10^0 to 10^{-20} dilution. Each sample was plated in triplicate and read 12 days after the beginning of the culture at 24ºC. Pipette tips were discarded after each dilution to avoid carrying adhered parasites from one well to another. Results are expressed as the negative log of the titer (*i.e.*, the dilution corresponding to the last positive well) adjusted per microgram of tissue.

2.11. Cytokine analysis

To measure IFN-γ, IL-4, and IL-10, the spleen cells (5 x 10^6 cells) were collected in the same time when the parasite load was evaluated in the animals and cells were stimulated with SLA *L. chagasi* (50 μg/mL), Fab5 or AbM (10 μg/mL, each one), in duplicate, on 24-well flat-bottomed plates (Nunc) for 48 h at 37ºC in 5% CO_2. The levels of IFN-γ, IL-4, and IL-10 were determined in the culture supernatants, using commercial kits (BD OptEIA™, Pharmingen), according to manufacturer instructions.

2.12. Statistical analysis

Statistical analysis was performed using the GraphPad Prism software (version 5.0 for Windows). The differences were evaluated by the one-way ANOVA analysis, followed by the post-Bonferroni test for comparisons among *in vitro* results and by the Tukey post-test for comparisons among *in vivo* experimental groups. All differences were considered significant when $P<0.05$. Data shown are representative of three separate experiments, which presented similar results.

3. RESULTS

3.1. *In vitro* efficacy of the Fab4 and Fab5 fractions from *A. blazei* water extract against *L. chagasi*

To analyze the in vitro effects in Leishmania viability of the different fractions obtained from AbM, stationary-phase promastigotes of *L. chagasi* were treated with different concentrations of the fractions to calculate the IC50 (Table 1). The Fab4 and Fab5 fractions presented the best results concerning the inhibition of the parasite viability, with IC50 values of 15.8 ± 1.2 and 13.0 ± 1.3 lg/mL, respectively, which were lower than those detected for the AbM. Since the IC50 values for Fab1, Fab2, and Fab3 were higher than those for the AbM (220.0 ± 2.8, 317.0 ± 3.3 and 218.0 ± 2.6 lg/mL, respectively) these fractions were discarded from further analysis. In a subsequent step, the toxicity of the AbM, Fab4, and the Fab5 fractions was evaluated. Murine macrophage viability (CC50) and the lysis of red blood cell suspensions (RBC50) were evaluated after treatment with the Agaricus preparations. The high CC50 and RBC50 values observed for AbM, as well as for both fractions (Table 2), demonstrated the low cytotoxicity of the preparations. When the selectivity index (SI = CC50/IC50) was calculated, the values encountered for the Fab4 and Fab5 fractions were higher than that obtained for the AbM, mainly due to the high IC50 value of this latter preparation. Since it has been postulated that candidates for new treatment regimens should present an SI value higher than 20 (Nwaka and Hudson, 2006), data from the present study indicate that the tested fractions can improve AbM properties.

The capacity of the AbM, as well as of the Fab4 and Fab5 fractions, to treat *in vitro* infected macrophages was also evaluated. Murine macrophages were infected with stationary-phase promastigotes of *L. chagasi* and later treated with the fractions (10 μg/mL, each one), for 48 h at 24 °C. The results showed that non-treated and infected macrophages presented 57% of the infection, with an average of 2.0 amastigotes per cell. The AbM treat- ment decreased the number of infected cells and the average number of amastigotes per cell (24.5 and 1.7 cells, respectively). Furthermore, treatment with Fab4 and Fab5 induced the highest decrease in the percentage of infected macrophages (4.0 and 1.7 cells, respectively) as well as in the average number of amastigotes per cell (0.8 and 0.4 cells, respectively) (Table 2). Comparing the data obtained with both fractions, the Fab5 displayed the best capacity to induce the *in vitro* reduction in the number of intracellular amastigotes.

Table 1- Analysis of *L. chagasi* promastigotes viability after treatment.

Substances	Concentration range (μg/mL)	IC_{50} (μg/mL)[a]
Fab1	10–200	220.0 ± 2.8
Fab2	10–200	317.0 ± 3.3
Fab3	10–200	218.0 ± 2.6
Fab4[b]	10–200	15.8 ± 1.2
Fab5[b]	10–200	13.0 ± 1.3
Water extract (AbM)	10–200	67.4 ± 1.7
Amphotericin B	0.1–10	1.0 ± 0.9

[a] Inhibition of *Leishmania* viability (50%) calculated by applying sigmoidal regression of the concentration–inhibition curves. Mean ± standard deviation is indicated.

[b] Statistical difference between the Fab4 and Fab5 groups and AbM group ($P < 0.001$).

Table 2- Cytotoxicity and treatment of infected macrophages.

Table 2
Cytotoxicity and treatment of infected macrophages[a].

Substances[b]	IC_{50}[c] (μg/mL)	CC_{50}[c] (μg/mL)	SI	RBC_{50}[c] (μg/mL)	Percentage of infected macrophages/number of amastigotes per cell[d]	Percentage of infected macrophages after treatment/number of amastigotes per cell[d]
Fab4	15.8 ± 1.2	662.8 ± 1.5	41.9	682.4 ± 1.8	57.0/2.0	4.0/0.8
Fab5	13.0 ± 1.3	674.4 ± 2.4	51.9	772.1 ± 2.0	57.0/2.0	1.7/0.4
AbM	67.4 ± 1.7	675.0 ± 2.1	10.0	770.5 ± 1.7	57.0/2.0	24.5/1.7
AmpB	1.0 ± 0.9	7.6 ± 2.0	8.8	N.D.[e]	57.0/2.0	4.0/2.8

[a] Murine peritoneal macrophages were employed in cytotoxicity and *in vitro* treatment assays: 4×10^5 and 5×10^5 cells per well, respectively. Erythrocyte suspensions (0* human red blood cells) were employed in hemolytic assays.

[b] For SI and RBC_{50} determination a concentration range of 10.0–200.0 μg/mL was employed for Fab4, Fab5 and the AbM groups. Amphotericin B (AmpB) was assayed at a concentration range of 0.1–10.0 μg/mL for SI determination. For treatment of infected macrophages, cells were incubated with the Fab4, Fab5, AbM or AmpB (10.0, 10.0, 50.0 and 1.0 μg/mL, respectively).

[c] The 50% inhibition of the macrophage viability (CC_{50}) or red blood cell lysis (RBC_{50}) were calculated by applying the sigmoidal regression of the concentration–inhibition curves.

[d] The percentage of infected macrophages and the number of amastigotes per cell were determined by counting 200 cell coverslips, in triplicate.

[e] N.D.: Not done.

3.2. *In vivo* treatment using the Fab5 or AbM against *L. chagasi*

Based on the *in vitro* results, Fab5 was selected for the *in vivo* experiments in order to treat BALB/c mice against *L. chagasi* infection. The fraction was applied to the animals before (in a chemo-prophylactic regimen) or after (in a therapeutic regimen) infection with stationary-phase promastigotes of *L. chagasi*. As controls, one independent mouse group was treated with AmpB at the 0-day of infection, while control group received distilled water. A dose–titration curve was performed in order to determine the concentration of Fab5 and AbM able to reduce significantly the tissue parasitism in treat mice. We observed that the concentration of 20 and 100 mg/kg/day, respectively, administered once a day, during 20 days post-infection, gave the best results in treating animals.

As shown in Fig. 1, mice from groups treated with the *Agaricus* preparations (AbM or Fab5), by the both treatment regimens–therapeutic (tAbM and tF5) versus chemo-prophylactic (cAbM and cF5), as compared to the control group that received distilled water, displayed significant reductions in the parasite burdens in all evaluated organs. In addition, parasite loads found in the three organs from the *Agaricus*-treated mice were significantly lower than those detected in mice treated with AmpB, except in the liver from ani- mals treated with the AbM preparations. Comparing the efficacy in reducing the parasite load in the animals treated with AbM or Fab5, animals treated with the Fab5 fraction presented a lower number of parasites than did those treated with the entire extract, although differences were not statistically significant. Interestingly, Fab5 chemo-prophylactic administration proved to be more effective than chemotherapeutic regimen, since mice included in the cF5 group showed lower parasite loads from all groups. This reduction is especially noticeable in the spleen, the internal organ that shows the chronic presence of the parasites after infection with viscerotropic *Leishmania* species, such as *L. chagasi*.

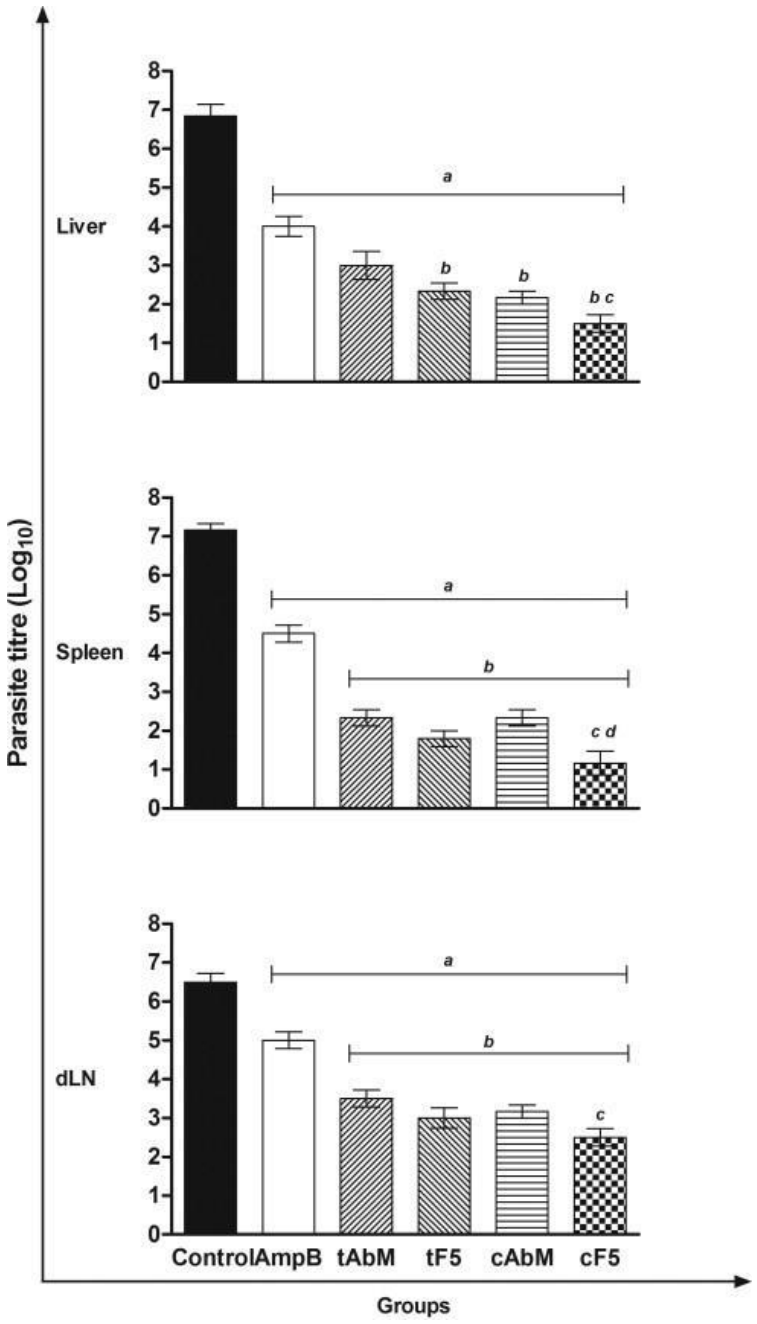

Figure 1- Evaluation of the *in vivo* treatment against *L. chagasi*. BALB/c mice (n = 12 per group) were infected in the right hind footpad with 107 stationary-phase promastigotes of L. chagasi. At week 10 post-infection, mice were euthanized, and the parasite load was determined in their livers, spleens, and dLN. Bars indicate the mean ± standard deviation (SD) of the groups. Significant differences among the groups are indicated by letters in the graphs: ‘‘a’’ indicates significant difference between the control group (distilled water) and the others; ‘‘b’’ indicates significant difference between the AmpB group and tAbM (AbM treatment), tF5 (Fab5 treatment), cAbM (AbM chemoprophylaxis), or cF5 (Fab5 chemoprophylaxis) groups; ‘‘c’’ indicates significant difference

between the indicated groups with respect the tAbM group, and ‘‘d’’ indicates significant difference between cF5 and tFab5 groups ($P < 0.05$).

3.3. Cellular immune response elicited after Fab5, AbM or AmpB treatments

To analyze the immune state induced by the administration of the Agaricus preparations, the production of IFN-γ (cytokine asso- ciated with protection) or IL-4 and IL-10 (cytokines associated with susceptibility) was determined in mice groups at 10 weeks post- infection. For this purpose, spleen cell cultures were stimulated with soluble *Leishmania* antigens (SLA, all groups), with AbM (for mice receiving AbM extract), or with Fab5 (for mice receiving the fraction).

Regarding the SLA induced production of cytokines, results from Fig. 2A shows that mice included in groups treated with the Agar- icus preparations (AbM or Fab5) presented higher levels of IFN-γ, and lower IL-4 and IL-10 levels than infected mice that received distilled water or that were treated with AmpB (except for the tAbM-treated mouse group, which displayed similar IFN-γ and IL-10 levels). Thus, a relation between the induction of Th1 response against parasite proteins and treatment success could be observed. Interestingly, as shown in Fig. 2B, a specific-Th1 response for the *Agaricus* preparations (AbM or Fab) could also be observed in mice treated with the fungal preparations after parasite infection, since the spleen cells from these mice were able to se- crete high levels of IFN-γ and moderate levels of IL-4 and IL-10 after stimulation with the respective *Agaricus* extracts. These data suggest that the administration of the *Agaricus* preparations in- duces a specific immune response with a Th1 profile.

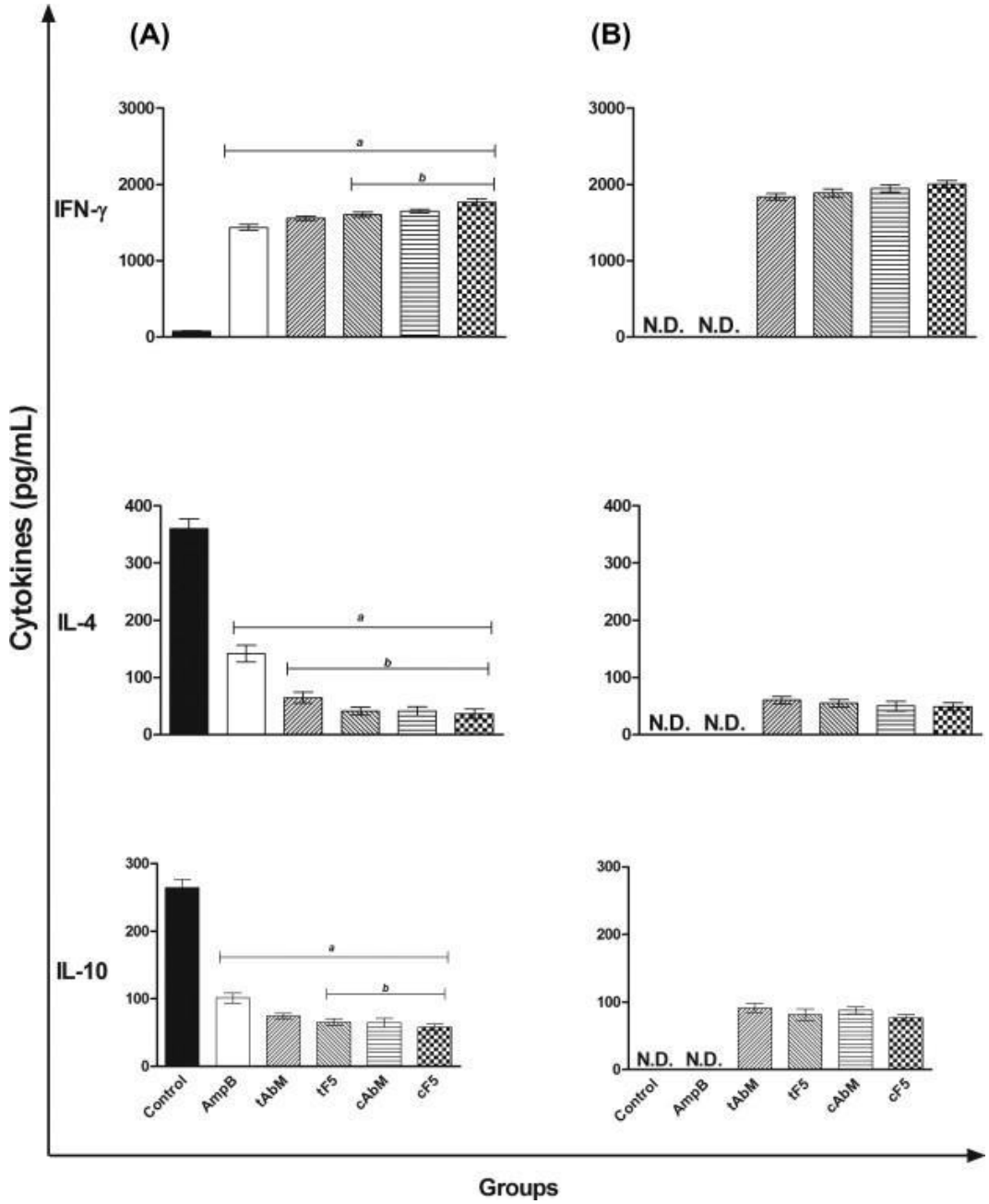

Figure 2- Production of cytokines by spleen cell cultures of treated and infected BALB/c mice. Single-cell suspensions were obtained from the spleens of non-treated or treated and infected mice, at 10 weeks post-infection. In panel A, cells were stimulated with SLA (50 lg/mL). In panel B, cultures were stimulated with Fab5 or AbM (10 lg/mL each), for 48 h at 37 °C, 5% CO2. IFN-c, IL-4, and IL-10 levels were measured in culture supernatants by ELISA. Bars indicate the mean ± standard deviation (SD) of the groups. Non- stimulated spleen cell cultures (background control) represented the basal production of the cytokines. Significant differences among the groups are indicated by letters in the graphs: ‘‘a’’ indicates significant difference between the control

group and the others; ‘‘b’’ indicates significant difference between the AmpB group and tAbM (AbM treatment), tFab5 (Fab5 treatment), cAbM (AbM chemoprophylaxis), and cFab5 (Fab5 chemoprophylaxis) groups ($P < 0.05$). ND: not detected.

To analyze the immune response elicited by the Agaricus preparations before infection, spleen cells from mice treated orally with AbM or Fab5 were *in vitro* cultured without stimulus or separately stimulated with the corresponding fungal product (AbM or Fab5), and the levels of IFN-γ, IL-4, and IL-10 was monitored in the supernatants. As can be deduced from the cytokine profiles shown in Fig. 3A for mice treated with the AbM and Fig. 3B for mice treated with the Fab5 fraction, the oral administration of both *Agaricus* preparations was able to induce a Th1 immune response mediated by IFN-γ production.

3.4. Chemical analysis of AbM and Fab5

Phytochemical screening carried out for AbM and Fab5 indicated the presence of glycoproteins, carbohydrates and tannins. The AbM and Fab5 profile showed six major peaks with retention times around 1.131, 1.250, 1.672, 2.425, 4.425 and 7.082 min. The UV spectra recorded on line for these peaks indicated similar wavelength absorption around 260 and 280 nm. Gallic acid (GA), epigallocatechin, catechin, epicatechin, gallocatechin and epigallocatechin gallate solutions were also analyzed as reference compound, employing the same chromatographic conditions. A GA standard solution, produced a peak with identical retention time and UV spectrum, retention time 2.425 min and UV spectra with wavelength maximum 271 nm (data not shown).

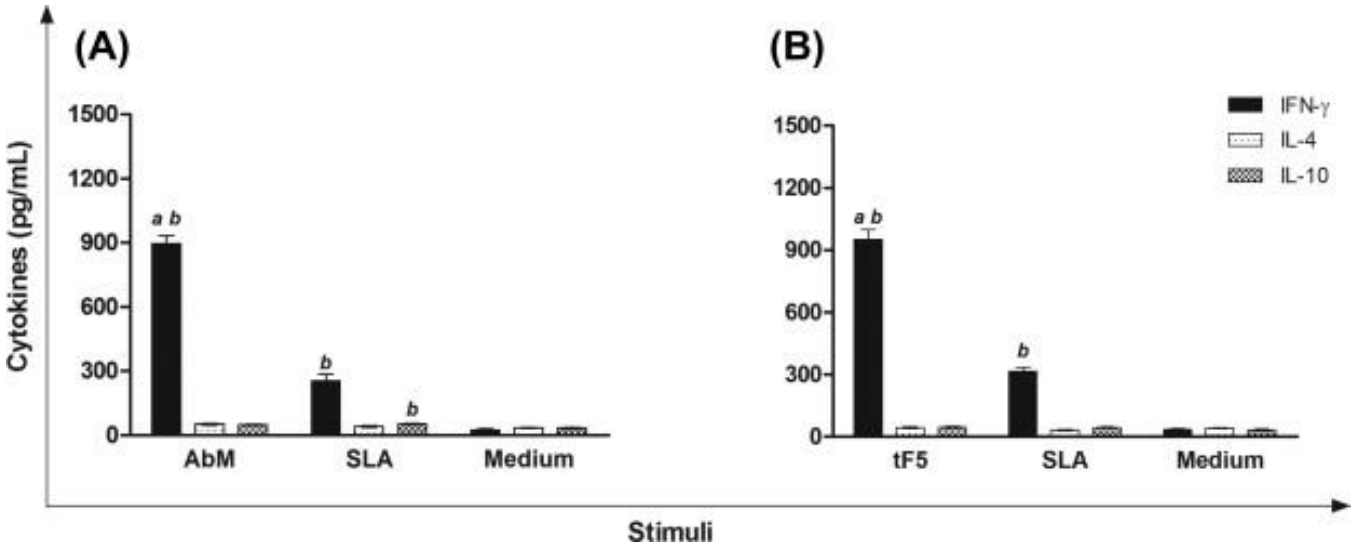

Figure 3- Production of cytokines by spleen cell cultures of treated BALB/c mice. Single-cell suspensions were obtained from the spleens of mice treated with AbM (in A) or Fab5 (in B), at 0-day (before) infection. Cells were stimulated with SLA *L. chagasi* (50 lg/mL) (all groups), AbM, or Fab5 fraction (10 lg/mL each one) for 48 h at 37 °C, 5% CO2. IFN-c, IL-4, and IL-10 levels were measured in culture supernatants by ELISA. Bars indicate the mean ± standard deviation (SD) of the groups. Non-stimulated spleen cell cultures (background control) represented the basal production of the cytokines. Statistical difference among the groups is indicated by letters in the graphs ($P < 0.05$). ‘‘a’’ indicates significant difference between the control group and the others; ‘‘b’’ indicates significant difference between the AmpB group and tAbM (AbM treatment), tFab5 (Fab5 treatment), cAbM (AbM chemoprophylaxis), and cFab5 (Fab5 chemoprophylaxis) groups.

4. DISCUSSION

The search for new products to treat VL has received consider- able attention. Since in a previously published work it was de- scribed that an aqueous extract of the A. blazei mushroom (AbM) had antileishmanial properties when assayed in vitro (Valadares et al., 2011) we decided to investigate in more detail these proper- ties using an in vivo model. Different fractions, segregated on the basis of their molecular weight were obtained and one of them, the

Fab5 was selected for the in vivo studies on the basis on its in vitro characteristics: the highest selectivity index (SI), the lowest cytotoxicity and the highest antileishmanial activity in cultured promastigotes and intracellular amastigotes from all studied fractions (Tables 1 and 2).

A highly stringent model for experimental chemotherapy was selected: BALB/c mice infected with a high infective inoculum (107 stationary promastigotes) of L. chagasi subcutaneously in the footpad. As it has been recently reported, this model of infection present an imbalance in their immune response that result in the establishment of the infection in the internal organs, including a chronic progressive parasite replication in the spleen (Oliveira et al., 2011b) which also occurs in classical VL models in which parasites were challenged intravenously (Carrión et al., 2006).

From the therapeutic regimen developed, in which a compara- tive evaluation of AbM or the F5b fraction and AmpB treatment was performed, Fab5 fraction and AbM were more effective, lead- ing to significant reductions in the parasite burdens than treatment with AmpB. Moreover, hepatic damage that resulted in increase in the ALT and AST hepatic enzymes levels was only observed in AmpB treated mice (data not shown), correlating to the data re- ported by Croft and Coombs (2003) that observed hepatic damage in BALB/c mice when AmpB was used. Previously reported data demonstrated that treatment with AbM reduced ALT and AST levels in a model of hepatic fibrosis in rats (Wu et al., 2011).

By comparing both administration regimens–therapeutic versus chemo-prophylactic it was observed that chemo-prophylactic therapeutic administration was more effective in reducing the de- gree of infection in the animals than therapeutics (Fig. 1). This higher efficacy can be related with the immune state induced by AbM or Fab5 administration, since these mice were able to mount an IFN-c specific response for the Fab5 or AbM, and low levels of IL-4 and IL-10, prior to infection. These observations are in agree- ment with previous reports showing that some components of the Agaricus mushroom are able to modulate the immune system inducing Th1 responses (Bouike et al., 2009; Tang et al., 2009). Thus, the Th1 response elicited by the pre-treatment may have helped to induce an non-specific cytokine milieu which is required to control of parasite replication in murine VL models, namely, induction of IFN-c and control of IL-4 and mainly IL-10 mediated responses (Stäger et al., 2010; Oliveira et al., 2011b).

After infection a correlation between the reduction of parasite burdens and the specific immune response elicited in the treated mice was found. Non-treated and infected BALB/c mice displayed IL-4 and IL-10 dependent responses against parasite proteins, the profile of cytokines associated with susceptibility to cutaneous and visceral leishmaniasis (Rhodes and Graham, 2002; Coelho et al., 2003; Basu et al., 2007). By contrast, mice that received AbM, Fab5, or AmpB, were able to produce higher levels of specific IFN-c and lower levels of IL-4 and IL-10, although the more pro- nounced Th1 immune response could be observed in the animals treated with Fab5 and AbM. These findings correlate with the ability of the *Agaricus* extracts to generate strong immune-stimulating effect, based on the activation of bone marrow-derived macro- phages (Sorimachi et al., 2001) and to induce the production of NO and inflammatory cytokines (IL-1, IL-6, IL-8, and TNF-a), but not of the anti-inflammatory IL-10 in human monocytes (Bernard- shaw et al., 2006) results in a reduction of the VL disease in this murine model.

Mushroom immune properties have been associated with polysaccharide fractions. Fungi b-glucans have been reported to activate leukocytes and can increase the phagocytic activity of the same cells, such as monocytes and granulocytes; the production of reac- tive oxygen intermediates; as well as the production of inflamma- tory mediators, such as cytokines and chemokines (Brown and Gordon, 2003; Bernardshaw et al., 2006; Bernardshaw et al., 2007). In this context, to provide additional information concerning the presence of chemical entities in the Fab5 fraction, a chemical characterization was performed, the results of which indicated the presence of polysaccharides, glycoproteins and tannins.

In relation to polysaccharide fractions, tannins represent a group of phenolic metabolites that exhibit a wide range of *in vitro* biochemical and pharmacological activities, including anti-viral and antimicrobial properties. Treatments of a broad spectrum of diseases with polyphenolic herbal medicines prompted interest in the evaluation of polyphenols as promising antileishmanial agents. Kolodziej and Kiderlen (2005) showed that infected macrophages augmented and prolonged activation of host defense mechanisms after incubation with different tannins classes and purposed that the effects of polyphenols can present beneficial effects in various infectious conditions, although in vivo experiments are essential to prove the therapeutic benefits of polyphenolic immunomodulators.

In conclusion, the data presented in this study showed the potential of the purified Fab5 fraction of AbM as a therapeutic alternative to treat visceral leishmaniasis. In addition, it can be suggested that Fab5 may be also employed in a chemo-prophylactic regimen.

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DISCUSSÃO

1 – Análise *in vitro* do potencial biotecnológico do extrato aquoso de *A. blazei* contra parasitas do gênero *Leishmania*

Com o propósito de avaliar a atividade leishmanicida do extrato do fungo *A. blazei* contra diferentes espécies de *Leishmania*, na caso, *L. amazonensis, L. major, L. chagasi*, em formas promastigotas e like-amastigota, foram realizados diferentes ensaios *in vitro*, para que se avaliasse a segurança do composto antes de iniciar os estudos pré-clínicos, ou mesmo se seriam viáveis. O extrato aquoso mostrou atividade leishmanicida contra diferentes espécies de *Leishmania* em ambas as formas do parasita, sendo que a atividade apresentada era independente da produção de NO. Além disso, o extrato apresentou uma baixa citotoxicidade em macrófagos murinos e nenhuma atividade hemolítica em glóbulos vermelhos humanos, revelando características desejáveis ao tratamento. O esquema utilizado nas avaliações é mostrado na figura 4.

Curiosamente, parasitas promastigotas previamente tratados com extrato aquoso de *A. blazei* se mostraram menos infectivos quando expostos a fagocitose por macrófaogs peritoneais murinos. O grupo do pesquisador Svodobodá em 1997 e em 2000 demonstrou a presença de lectinas em ambas as formas promastigotas e amastigotas de diferentes espécies de *Leishmania*, além de revelar um papel das lectinas na multiplicação e sobrevência desses parasitas. O alto teor de carboidratos presentes no extrato aquoso pode interagir com as moléculas de lectinas presentes na superfície dos parasitas e reduzir sua infectividade por impedimento estérico com receptores de macrófagos essenciais para a invasão, como MFR (mannose-fucose recptor) ou CR1.

2 – Análise da eficácia terapêutica de preparações à base de *Agaricus blazei* e seu potencial para uso em diferentes formas clínicas de leishmaniose.

Testes pré-clínicos em camundongos BALB/c infectados com *L. amazonensis* foram eficazes no controle de progressão da lesão e na replicação do parasita, mesmo quando comparado ao tratamento referência, com AmpB. Além disso, pudemos detectar a eliminação

completa de parasitas no baço dos animais tratados na 10ª semana, condição essa mantida até a 14ª semana após a infecção, o que sugere que o tratamento oral impediu a disseminação de parasitas do local da infecção para os órgãos internos mantendo a parasitemia confinada e restrita ao linfonodo. Essa restrição pode ter contribuído para a enorme redução de parasitas, uma vez que o confinamento de parasitas causadores de forma clínica cutânea nos linfonodos pode estar relacionada com a memória imunológica nessas infecções, sendo que os antígenos oriundos dessa baixa parasitemia poderiam servir como estímulos para manter a população de células T de memória efetora (Kaye & Scott 2011). Isto é especialmente importante se for considerado que a doença causada por espécies de *Leishmania* do Novo Mundo pode ser considerada uma doença sistêmica. Além disso, tal eficácia foi revelada nas duas estratégias de tratamento abordadas na tese, tanto preventiva quanto terapêutica.

Após os experimentos pré-clínicos em modelos de leishmaniose cutânea, foi realizada triagem de frações semi-purificadas, de maneira a selecionar a fração de melhor custo benefício e alta atividade leishmanicida. Com isso, tanto o extrato aquoso de *A. blazei* quanto sua fração F.a.b 5, apresentaram elevada eficácia terapêutica, mas também profilática, no modelo visceral causado pela infecção por *L. chagasi*. Aponte-se que a estratégia quimioprofilática com a Fab5 revelou ser o tratamento mais efetivo, mesmo comparando com o tratamento referência com anfotericina B, mantendo, também, normais os níveis séricos das enzimas hepáticas AST e ALT (dados não mostrados).

Em ambos os modelos pré-clínicos de leishmaniose testados, tanto cutânea quanto visceral, os tratamentos com os produtos de *A. blazei* (extrato aquoso mas também frações semi-purificadas) desencadearam uma imunomodulação no sentido de polarizar a resposta imune para o perfil Th1. Parasitas de diferentes espécies têm a capacidade de subverter a resposta imunológica do hospedeiro de maneira a se beneficiar desta alteração, sendo que o aumento da citocina imunossupressora IL-10 tem um papel crucial na susceptibilidade à infecção por espécies de Leishmania que desenvolvem formas clínicas cutâneas (como *L. major*). O aumento de níveis da forma ativa de TGF-β está relacionado com a evolução da forma clínica visceral (como *L.i.c*), tanto em humanos quanto em camundongos (Kaye & Scott 2011, Grant et al. 2003). Também foram reportadas propriedades imunomodulatórias de agentes quimioterápicos para Leishmaioses, como antimoniais de sódio (infecção de camundongos BALB/c com *L. donovani*), que podem aumentar os níveis de ROS, NO e TNF-

alfa; anfotericina B (infecção de Hamister com *L.i.c*), o qual aumenta os níveis de IL-12,TNF-alfa e iNOs, mas também reduz níveis de IL-10, TGF-β e também miltefosina (infecção em BALB/c por *L. donovani*), a qual pode estimular linfócitos T, aumentar os níveis de INF-gama e também estimular macrófagos. Tirei uma frase por ser de difícil compreensão

No caso do tratamento com produtos do *Agaricus blazei*, os resultados obtidos na tese revelaram uma polarização da resposta Th1 em animais infectados, mas também em animais não infectados, revelando que o tratamento oral, por si só, foi capaz de causar a polarização da resposta Th1 com aumento de níveis de INF-gama e redução dos níveis de IL-4. Tal fato está de acordo com o proposto em diversos trabalhos de que o extrato de *A. blazei* induz um perfil Th1 e que provavelmente a via oral é importante para tal processo. Bouike et al 2010 revelaram um papel do H2O2 produzido por células epiteliais intestinais, após o tratamento com extrato aquoso de *A. blazei*, como segundo mensageiro na ativação de macrófagos e células dendríticas (Hetland et al. 2011, Mizuno & Nishitani 2013). Em modelos de estudos com células intestinais, como células CaCo2, podem revelar se realmente a ativação de macrófagos através do tratamento de *A. blazei* ou suas frações semi-purificadas, necessita da participação da via oral ou de células intestinais. Tal fato poderia explicar a ativação de macrófagos via produção de NO apenas nos tratamentos *in vivo* e não nos experimentos *in vitro*, observação também feita por Volmanet et al. 2010.

Outra resultado interessante observado foi que não houve alteração das enzimas hepáticas ALT e AST, refletindo uma possível propriedade hapatoprotetora de tal fungo. A hepatoproteção causada pela administração de *A. blazei* também foi relatada em modelo de lesão de fígado de ratos induzida por paracetamol (Soares et al 2013), e por CCl4 (Wu et al. 2011). Tal propriedade pode ser explorada também pelo uso dos produtos oriundos do *A. blazei* em conjunto com tratamentos convencionais, uma vez que - especialmente em formas clínicas viscerais - a lesão hepática pode ser fatal ou até mesmo impedir a continuação de algumas opções de tratamento, como antimoniais e anfotericina B, já que são hepatotóxicos por si só.

Com o intuito de se triar e selecionar frações que mantenham as principais características dos tratamentos aqui propostos, como baixa toxicidade, atividade antiparasitária, imunomodulação e hepatoproteção, tem-se a perspectiva de testar diferentes frações semi-purificadas, no intuito de se estabelecer qual proporção apresenta o melhor

sinergismo de ação pelo melhor custo benefício para seguir com o escalonamento e desenvolvimento de estratégia de produto, seja veterinário ou humano.

Através dos resultados obtidos, pudemos verificar a atividade leishmanicida do fungo *A. blazei* contra diferentes espécies de *Leishmania* (*L. amazonensis*, *L. major* e *L. chagasi*) nas formas promastigotas e amastigotas axênicas, bem como nas formas amastigotas intracelulares. Também pudemos observar que o extrato aquoso de *A. blazei* não apresentou toxicidade em macrófagos murinos e em eritrócitos humanos. O estudo também mostrou que a atividade leishmanicida do extrato de *A. blazei* não foi mediada apenas pela produção de NO em macrófagos infectados, mas sim pela ação direta de seus compostos nos parasitas internalizados em macrófagos.

Testes pré-clínicos em camundongos BALB/c infectados com *L. amazonensis* mostraram a eficácia preventiva e terapêutica do extrato do fungo baseado na redução significativa do inchaço da pata e da carga parasitária, bem como o desenvolvimento de uma resposta imune Th1 mostrada pela produção de IFN-gama e NO pelas culturas esplênicas estimuladas *ex vivo*. Outro fator importante foi a ausência de toxicidade nos animais tratados, e a normalização dos níveis de transaminases quando comparados aos níveis encontrados nos demais animais experimentais.

O extrato aquoso de *A. blazei* e sua fração F.a.b 5, previamente selecionada por apresentar melhor rendimento de produção e elevada atividade antiparasitária *in vitro*, também apresentaram elevada eficácia terapêutica e profilática na infecção causada por *L. chagasi*. Também semelhante os modelos infectados com *L. amazonensis*, os animais infectados com *L.i.c.* e submetidos a diferentes tratamentos obtidos a partir do *A. blazei* revelaram um aumento na produção de IFN-gama e uma redução de IL-4, sugerindo uma polarização do perfil imunológico Th1.

CONCLUSÕES

Através dos resultados obtidos, pudemos verificar a atividade leishmanicida do fungo *A. blazei* contra diferentes espécies de *Leishmania* (*L. amazonensis*, *L. major* e *L. chagasi*) nas formas promastigotas e amastigotas axênicas, bem como nas formas amastigotas intracelulares. Também pudemos observar que o extrato aquoso de *A. blazei* não apresentou

toxicidade em macrófagos murinos e em eritrócitos humanos. O estudo também mostrou que a atividade leishmanicida do extrato de *A. blazei* não foi mediada apenas pela produção de NO em macrófagos infectados, mas sim pela ação direta de seus compostos nos parasitas internalizados em macrófagos.

Testes pré-clínicos em camundongos BALB/c infectados com *L. amazonensis* mostraram a eficácia preventiva e terapêutica do extrato do fungo baseado na redução significativa do inchaço da pata e da carga parasitária, bem como o desenvolvimento de uma resposta immune Th1 primada pela produção de IFN-gama e NO pelas culturas esplênicas estimuladas *ex vivo*. Outro fator importante foi a ausência de toxicidade nos animais tratados, e a normalização dos níveis de transaminases quando comparados aos níveis encontrados nos demais animais experimentais.

O extrato aquoso de *A. blazei* e sua fração F.a.b 5, previamente selecionada por apresentar melhor rendimento de produção e elevada atividade antiparasitária *in vitro*, também apresentaram elevada eficácia terapêutica e profilática na infecção causada por *L. chagasi*. Também semelhante os modelos infectados com *L. amazonensis*, os animais infectados com *L.i.c.* e submetidos a diferentes tratamentos obtifdos a partir do *A. blazei* revelaram um aumento na produção de IFN-gama e uma redução de IL-4, sugerindo uma polarização do perfil imunológico Th1.

Capítulo 4 – ARTIGO EM PREPARAÇÃO

Involvement of the non-inflammasome forming Nucleotide-binding domain leucine-rich repeat protein 12 (Nlrp12) in visceral leishmaniasis (VL).

CAPÍTULO 4: Trabalho desenvolvido durante o doutorado sanduíche no laboratório da profa. Dra. Mary E. Wilson, na Universidade de Iowa, Estados Unidos, que será apresentado na forma como será submetido para publicação.

Título do artigo:

Involvement of the non-inflammasome forming Nucleotide-binding domain leucine-rich repeat protein 12 (Nlrp12) in visceral leishmaniasis (VL).

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ABSTRACT

Leishmania infantum chagasi (Lic) causes VL, with suppression of type 1 immune responses. The NLR proteins include >20 cytosolic proteins that regulate inflammation and immunity. Activation of three NLRs or AIM2 can cause assembly of an inflammasome leading to IL-1β and IL-18 release. Functions of non-inflammasome forming NLRs are not as well understood. **We hypothesized that NLR proteins influence the course of VL by modifying the localized inflammatory response to *Lic*.** We screened for NLR effects by infecting NLR pathway gene knockout or wild type (WT) mice with *Lic* coexpressing luciferase and mCherry. Progressive parasite expansion was monitored by in vivo imaging (IVIS), qPCR and, Luciferase assay. The screens suggested involvement of the non-inflammasome forming Nlrp12 in progression of VL. *Lic* parasite loads expanded early (day 28) but were controlled in WT mice, whereas *Lic* continued to expand and were 2-fold higher than WT on day 56 of *Nlrp12*$^{-/-}$ infection. Consistently, liver-derived infiltrating cells from *Nlrp12*$^{-/-}$ mice released less antigen-induced IFNgamma than WT cells on infection day 56 (24 vs. 41 pg/mL). Flow Cytometry showed inflammatory monocytes expanded on day 28 in WT but not *Nlrp12-/-* mice, preceding parasite clearance from WT. Instead, resident macrophages expanded in *Nlrp12-/-* mice in parallel with the late expanding parasite load (day 56). The kinetics of monocyte derived dendritic cell (MNDC) recruitment paralleled parasite load, with recruitment at 28 days in WT but recruitment at 56 days in *Nlrp12-/-* mice. These data suggest that Nlrp12 plays a protective role in VL, associated with recruitment of both inflammatory monocytes and MNDCs at the time of peak parasite growth, followed by parasite clearance. Infiltration of inflammatory monocytes is impaired in the absence of Nlrp12, leading to delayed MNDC influx, impaired IFN-gamma, and expansion of resident macrophages, which permit parasite growth.

1. INTRODUCTION

In mammals, multiple recognition systems have coevolved to preserve normal interactions with the commensal flora and to initiate immune responses to invading pathogens and perturbations in tissue homeostasis. Endosomal and extracellular Toll-like receptors recognize mainly pathogen-associated molecular patterns found in microbes. Meanwhile, a

multitude of cytosolic receptors recognize not only intracellular pathogen-associated molecular patterns but also the host-derived signals known as ‘damage-associated molecular patterns’. The cooperation between these compartmentalized surveillance systems allows organisms to sense and respond to a large number of infectious and sterile insults to the host.

Nod-like receptors (NLRs) are cytosolic pattern-recognition receptors that were initially proposed to regulate inflammation through apoptosis, on the basis of their structural homology to proteins involved in defense against infection in plants (NB-LRR proteins) and to the apoptosis-activating factor APAF-1. However, a decade ago that initial concept was modified with the discovery of a large protein complex composed of the NLR protein NLRP1, the adaptor ASC and the proinflammatory caspases 1 and 5 (Martinon et al. 2002). This large complex was called the ‘inflammasome’, as it was demonstrated to be required for activation of the proinflammatory cytokine interleukin 1β (IL-1β). Subsequently, several other sensor proteins of the NLR family and PYHIN family (pyrin- and HIN-200 domain–containing proteins) have been shown to form inflammasomes in response to a wide variety of damage-associated and pathogen-associated molecular patterns (Strowig et al. 2012). (Fig. **1).** Today, inflammasomes are recognized as one of the cornerstones of the intracellular surveillance system.

Although the first decade of research in the inflammasome field shed light on the bio-chemical principles of inflammasome assembly, specificity and function of inflammasomes, exciting new evidence has placed inflammasomes and NLR proteins at the center stage of complex diseases (metabolic syndrome and carcinogenesis) and physiological processes (regulation of intestinal microbial ecology). (Henoa-Mejia et a. 2012).

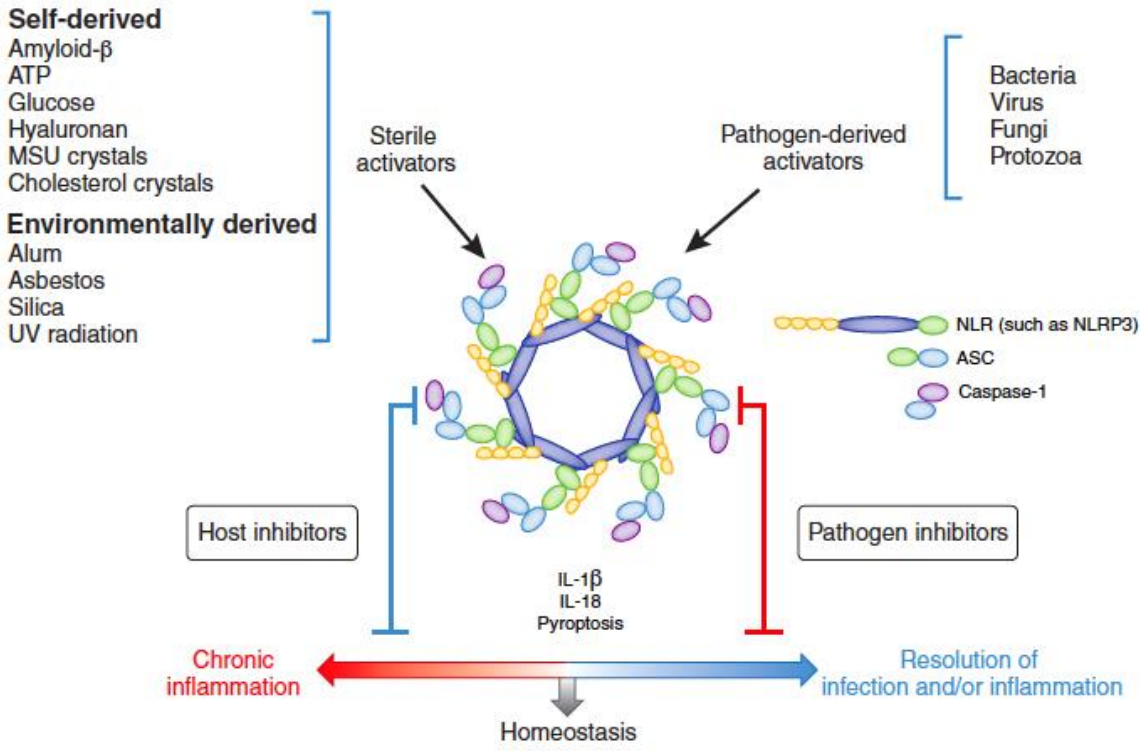

Figure 1- Inflammasome activity regulates homeostatic processes and inflammation during infection and tissue injury. During infection or injury, inflammasomes are directly or indirectly activated by a wide array of danger-associated molecular patterns. The initial event leads to activation of caspase-1, release of IL-1β and IL-18, and sometimes pyroptosis. Release of IL-1 β and IL-18 results in recruitment of effector cell populations of the immune response and tissue repair. Under normal circumstances, activation of the inflammasomes culminates in the resolution of infection or inflammation and contributes to homeostatic processes (that is, intestinal microbial ecology and regeneration of epithelial cells after injury). However, perpetuation of inflammasome activation can lead to chronic inflammatory diseases. Pathogen-derived inhibitors block inflammasome activation and thus the resolution of infection, whereas host-derived inflammasome inhibitors prevent the perpetuation of chronic inflammation. UV, ultraviolet (Strowig et al. 2012).

NLR proteins are evolutionarily related to plant NB–LRR proteins (Chisholm et al. 2006, Jones et al. 2006) also referred to as disease-resistance or R proteins, for their crucial function in host defense against infection. NLRs are also reminiscent of apoptosis-activating factor (APAF)-1, which assembles the apoptosome following cytochrome c release from the mitochondria, and initiates apoptosis by recruiting and activating caspase-9 (Bratton et al. 2010). NLRs are characterized by a tripartite structure composed of an invariant central domain

that mediates nucleotide binding and oligomerization referred to as a NACHT, NOD or NBS domain, a C-terminal LRR domain that senses NLR agonists, but exerts auto-inhibitory effects in their absence (Riedl et al. 2007), and a variable N-terminal region that is required for homotypic protein-protein interactions. The human NLR family consists of 22 members, classified into 4 subfamilies, namely the NLRA, NLRB, NLRC and NLRP subfamilies, on the basis of their N-terminal domain configuration (Ting et al. 2008) (Figure 2). NLRA contains an acidic transactivation domain; NLRB, a baculoviral inhibitory repeat domain; NLRC, a caspase-recruitment and activation domain (CARD); and NLRP, a pyrin domain (PYD). Notably, the CARD and PYD belong to the death-fold structural family, which also encompasses the death domain and death-effector domain, consisting of a tertiary structure commonly found in proteins involved in apoptosis or inflammation-related processes (Lahm et al. 2003).

It is now apparent that only a subset of NLR proteins forms inflammasome complexes, i.e. NLRP1, NLRP3, and NLRC4. Other NLR proteins have been found to be involved in innate immune responses, although their roles in inflammasome formation or inhibition remain elusive or controversial (Zambetti et al. 2012, Allen et al. 2013, Lich et al. 2007).

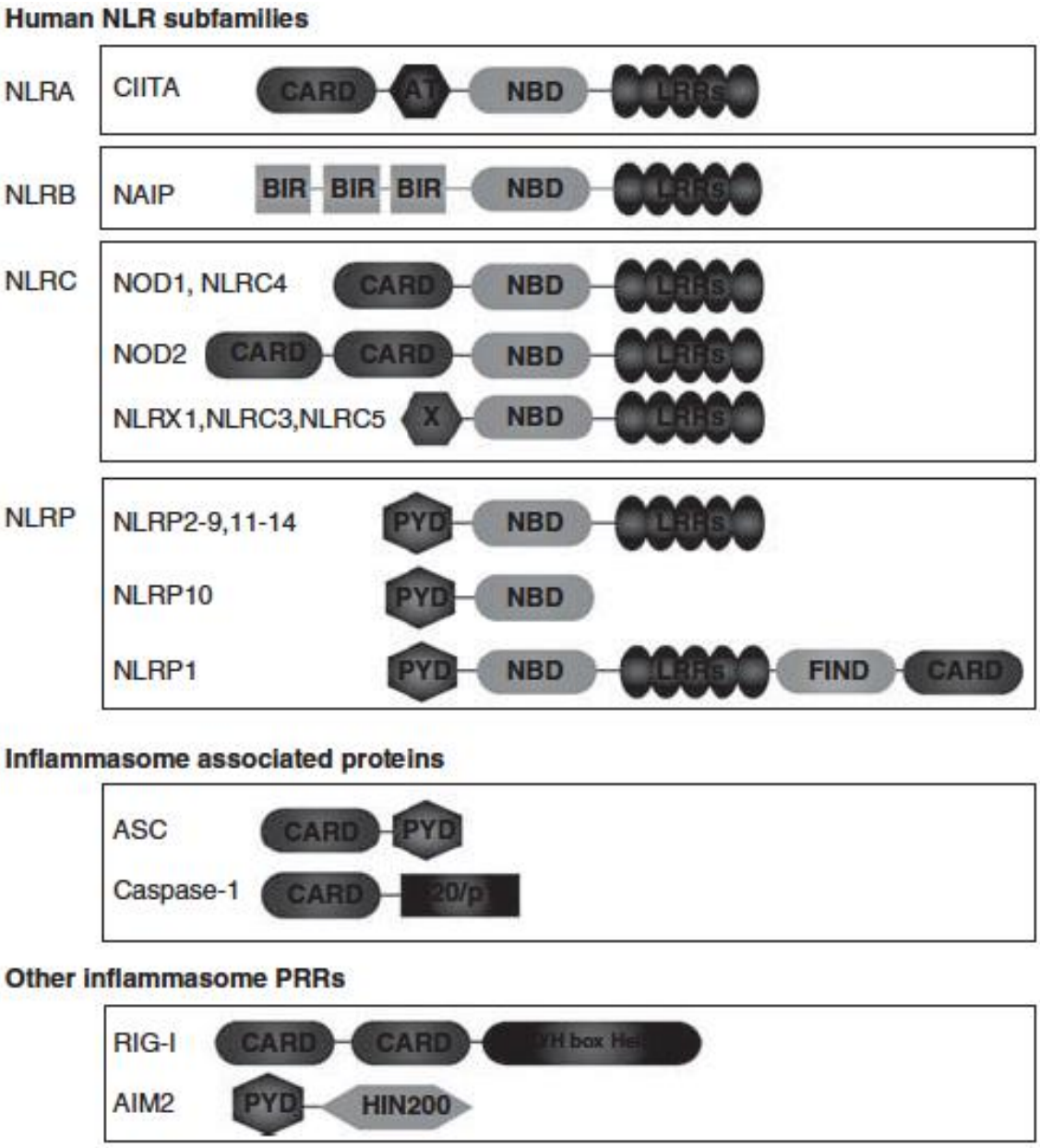

Figure 2- The human NLR family and inflammasome-associated proteins. There are 22 human NLRs characterized by a central nucleotide-binding domain (NB). The NLR family can be further classified into four subfamilies, depending on the protein's N-terminal domain. CARD and PYD domains enable interaction with caspase-1 or the adaptor ASC, allowing assembly of the inflammasome. The pattern-recognition receptors (PRRs) AIM2 and RIG-I are also capable of forming inflammasomes (Dagenais et al 2012).

The innate immune system is the first line of defense against pathogens and is initiated by genome-encoded pattern recognition receptors (PRRs), which respond to invading

microbes. Upon infection, PRRs recognize microbial pathogen-associated molecular patterns (PAMPs) and endogenous danger-associated molecular patterns (DAMPs), leading to the activation of host defense pathways that result in the clearance of the infection. Toll-like receptors (TLRs) are a well-defined group of membrane-bound extracellular and endosomal receptors that play an important role in pathogen detection. A relatively new and interesting PRR-containing complex in innate immunity is the inflammasome, a multi-protein complex that acts as a platform for the activation of the pro-inflammatory caspase-1; the active form of which then proteolytically cleaves the cytosolic-sequestering leader sequence from pro-IL-1 β, pro-IL-18, and pro-IL-33 (Franchi et al. 2012, Davis et al. 2011) to generate mature cytokines which are released from the cell to mediate downstream inflammatory effects.

Inflammasomes are constructed of pro-caspase-1 and proteins in the cytosol; NLR (nucleotide-binding domain and leucine-rich repeat-containing family protein) NLRP1, NLRP3 or NLRC4, or AIM2. Inflammasomes require the adapter protein ASC that mediates interaction between the NLR or AIM2 and caspase- 1. Members of the entire NLR family are comprised of a pyrin-domain (or an amino- terminal caspase-activation and recruitment domain (CARD), a nucleotide-binding and oligomerization domain (NOD), and leucine-rich repeats (LRRs) (Davis et al. 2011) that are responsible for the recognition of PAMPs or other signals (Figure 3). Inflammasome-mediated cytokine release follows a multi-step activation pathway. Prior to inflammasome assembly, a priming step (often a TLR ligand) results in NF-kB-dependent upregulation of the inactive pro-forms of IL-1β and IL-18 and also of some NLRs like NLRP3 (Bauenfeind et al. 2009). There is a second activation step triggered by recognition of intracellular "danger" signal, which results in inflammasome formation through AIM2 or one of the 3 above mentioned NLR proteins (Figure 3). Recently, a 3-step activation pathway has been described for some Gram-negative bacteria, that involves caspase-11 and TLR4/TRIF (Broz et al. 2012, Rathinan et al. 2012). It should be noted that some cells might have a simpler activation process due to higher basal levels of the pro-forms of caspase-1 and/or pro-cytokines (Netea et al. 2009). Also, some signals serve as both the priming and the activation signal, such as *Candida albicans* (REF FAYYAZ). The inflammasome-forming NLRSs in general have been well characterized, and can play roles in bacterial recognition (NLRC4, NLRP3, AIM2). In contrast, details are emerging for the non-inflammasome forming NLRs (e.g., NLRP6, NLRP7, NLRP12). Some have been claimed to behave as

negative regulators of inflammasomes, although their relation to bacterial infection has yet to be defined (Rathinan et al. 2012). Inflammasome activation has also been shown to initate a special pathway of cell death called pyroptosis (Miao et al 2011, Gregory et al. 2013).

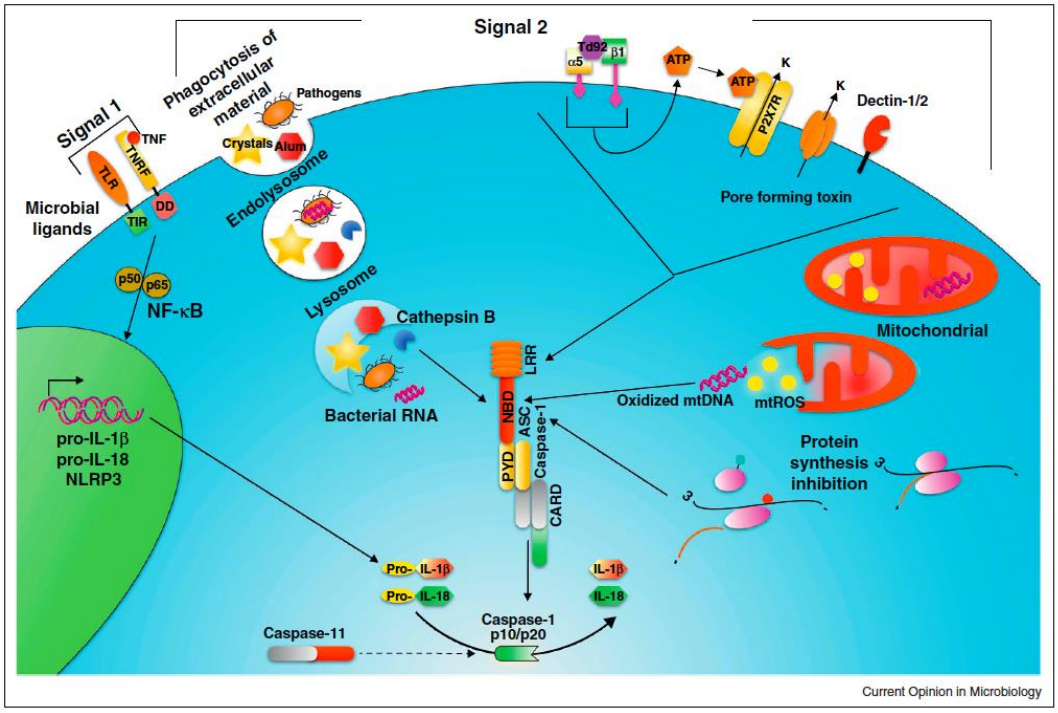

Figure 3- Model of NLRP3 activation. Activation of caspase-1 by the NLRP3 inflammasome is a multi-signal process. Signal 1 occurs when TNF or a TLR ligand binds its cognate receptor resulting in the translocation of NF-kB into the nucleus where expression of NLRP3 and the immature (pro-) forms of IL-1 β and IL-18 are induced. Signal 2 is the activation of NLRP3 resulting in recruitment and cleavage of pro-caspase-1 to its active form leading to cleavage of the immature inflammatory cytokines. At least three distinct NLRP3 activation pathways have been identified. Phagocytosis of extracellular particulates and pathogens results in lysosomal destabilization and release of cathepsin B and bacterial mRNA, which trigger NLRP3 activation. A decrease in intracellular K+ has been shown to result in activation of NLRP3. K+ efflux occurs by engagement of extracellular ATP with the P2X7R or directly through bacterial pore-forming toxins. ROS generated during mitochondrial damage and oxidized mitochondrial DNA (mtDNA) produced during apoptosis lead to activation of NLRP3. Td92, a surface protein of *Treponema denticola*, can interact with the a5b1 integrin resulting in ATP release and K+ efflux. Inhibition of ribosomal function and protein synthesis can also direct NLRP3 activation, and this mechanism may involve lysosomal destabilization, K+ efflux and ROS. Caspase-11 has been defined upstream of caspase-1 during NLRP3 inflammasome activation (Gregory et al. 2013).

There is evidence supporting a function in bacterial recognition for several NLRs. These include NOD1/2 (recog- nizing peptidoglycan fragments) (Martinon et al., 2009), NLRP1 (sensing anthrax lethal toxin) (Averette et al., 2009), NLRP3 (activated by exposure to many pathogens, bacterial RNA, toxins, and crystal structures) (Davis et al., 2011; Duewell et al., 2010; Halle et al., 2008; Hornung et al., 2008; Kanneganti et al., 2006; Sander et al., 2011), NLRC4 (sensing of *Salmonella,* intracellular flagellin and bacterial type III secretion rod proteins) (Franchi et al., 2006; Miao et al., 2010), and Naip5 (promoting resistance to *Legionella)* (Kofoed and Vance, 2011; Molofsky et al., 2006; Ren et al., 2006). Recent results also suggested a role for NLRP6 in maintenance of bacterial homeostasis in the colon and for NLRP7 in the recognition of lipoproteins (Khare et al., 2012). NLRP12 (also called Nalp12, Monarch-1, and Pypaf-7) was the first NLR shown in biochemical assays to interact with the adaptor protein Asc to form an active IL-1b-maturing inflammasome (Khare et al., 2012).

The bulk of NLR publications have sought to characterize a sub-group of NLRs that function in the formation of a multi-protein complex termed the inflammasome, which includes a specific NLR, the adaptor protein PYCARD and Caspase-1. An inflammasome forms following the activation of a particular NLR by a specific danger signal, which ultimately results in the activation of caspase-1 and the subsequent post- translational cleavage of pro-IL-1b and pro-IL-18 into their active forms (Allen et al. 2013).

Unlike the majority of characterized proinflammatory NLRs, recent studies have identified a novel sub-group of NLRs that have the ability to attenuate inflammation (Lich et al. 2013). The exact mechanism underlying this negative regulation is an area of active investigation; however, it appears that most of these NLRs function by inhibiting components of NF-kB signaling. The NLR, NLRP12 (formally known as MONARCH-1 or PYPAF7), is a prototypical member of this sub-group that functions as a negative regulator of the immune system through the attenuation of non- canonical NF-kB signaling and p52-dependent chemokine expression. This suppression is facilitated by the association of NLRP12 with the proteasome and the subsequent proteasome- dependent degradation of NF-kB inducing kinase (NIK) (Lich et al. 2007).

The role of NLRP12 in innate immunity has remained unclear. Both inflammatory and inhibitory functions have been suggested, as has a role in hypersensitivity (Allen et al., 2012; Arthur et al., 2010). Interestingly, as for NLRP3, mutations in NLRP12 are linked to hereditary

inflammatory disease (Jéru et al., 2008), and mutations may lead to increased Asc speckle formation and caspase-1 activity (Jéru et al., 2011B). It has been reported that patients carrying NLRP12 mutations and increased inflammasome activation have been successfully treated with anti-IL-1 therapy, similar to patients containing mutations in NLRP3 (Jéru et al., 2011A; Lachmann et al. 2009). Nonetheless a direct role for NLRP12 in inflammasome activation has not been found, and these effects are in some manner indirect.

It has been shown that murine Nlrp12, an NLR linked to atopic dermatitis and hereditary periodic fever in humans, is prominently expressed in dendritic cells (DCs) and neutrophils. Nlrp12-deficient mice exhibit attenuated inflammatory responses in two models of contact hypersensitivity that exhibit features of allergic dermatitis. This cannot be attributed to defective Ag processing/presentation, inflammasome activation, or measurable changes in other inflammatory cytokines. Rather, Nlrp12-/- DCs display a significantly reduced capacity to migrate to draining lymph nodes in vivo. Both DCs and neutrophils fail to respond to chemokines *in vitro*. These findings suggest that NLRP12 is important in maintaining neutrophils and peripheral DCs in a migration-competent state in experimental animal models (Arthur et al. 2010).

It has also been shown that the NLRP12 inflammasome is an important regulator controlling IL-18 and IL-1β production after *Y. pestis* infection. NLRP12-deficient mice were more susceptible to bacterial challenge than wild type control mice on a C57BL/6 background. NLRP12 also directed IFN-γ production via induction of IL-18, but had minimal effect on signaling to the transcription factor NF-kB. These studies suggest a role for NLRP12 in host resistance against pathogens. Minimizing NLRP12-associated inflammasome activation may have been a central factor in evolution of the high virulence of *Y. pestis* (Vladimer et al. 2012).

Parasites of the genus *Leishmania* are the causative agents of leishmaniasis in humans, a disease that affects more than 12 million people worldwide. These parasites replicate intracellularly in macrophages, and the primary mechanisms underlying host resistance involve the production of nitric oxide (NO). The generation of an appropriate immune response, which requires an effective innate immune recognition of parasites, is essential for human resistance against infectious diseases, including leishmaniasis. Although MyD88-dependent Toll-like receptor (TLR) signaling has been reported to be important for parasite recognition (Werner-Felmayer et al. 1990, Debus et al. 2003), species of *Leishmania* are known to be

immunologically silent and bypass recognition by innate immune receptors during infection (Gregory et al. 2005, Xin et al. 2008). The role of NLR proteins, whether inflammasome-forming or non-inflammasome-forming, in infectious diseases caused by protozoan parasites is still unclear. Sjio et al. 2009 demonstrated that NLRP3, ASC and caspase-1 are essential for the hemozoin-induced inflammatory responses during *Plasmodium chibaudi* infection, and this response is mediated by Syk and Lyn. They also demonstrated that the absence of NLRP3 or IL-1 β augmented survival to malaria caused by *P. chabaudi* (Shio et al. 2009). Witola et al. 2011 demonstrated a role for the NALP1 inflammasome during infection with *T. gondii*, using a genetically engineered human monocytic cell line for NALP1 gene knockdown by RNA interference. NALP1 silencing attenuated progression of *T. gondii* infection, with accelerated host cell death and eventual cell disintegration. In line with this observation, upregulation of the proinflammatory cytokines interleukin-1beta (IL-1 β), IL-18, and IL-12 upon *T. gondii* infection was not observed in monocytic cells with NALP1 knockdown. These findings suggest that the NALP1 inflammasome is critical for mediating innate immune responses to *T. gondii* infection and pathogenesis (Witola et al. 2011). The only study involving inflammasome response and Leishmania parasites were performed by Lima-Junior et al. 2013, and in this study the group showed that the Nlrp3 inflammasome is activated in response to *Leishmania* infection and is important for the restriction of parasite replication both in macrophages and *in vivo* as demonstrated through the infection of inflammasome-deficient mice with *Leishmania amazonensis, Leishmania braziliensis* and *Leishmania infantum chagasi.* Inflammasome-driven interleukin-1β (IL-1β) production facilitated host resistance to infection, as signaling through IL-1 receptor (IL-1R) and MyD88 was necessary and sufficient to trigger inducible nitric oxide synthase (NOS2)-mediated production of NO. However this study was the first to describe the involvement of the inflammasome forming NLRP3, the role of nonforming NLR proteins, like NLRP12, in *Leishmania* infection is completely unknown. So we aimed this study to reveal the relation of NLRP12 in *Leishmania chagasi* infection.

2. MATERIAL AND METHODS

2.1 Mice

Female BALB/c mice (4 to 6 weeks old) were purchased from Harlan Breeders. Studies were approved by the Animal Care and Use Committees of the University of Iowa and the Iowa City Veterans' Affairs Medical Center. NLRP12$^{-/-}$ or WT control mice on a C57BL6 background were infected with 1x10^6 metacyclic Luc-mCherry expressing *L. infantum chagasi* through a tail vein. The course of infection and the infection burden were monitored by the intensity of the luciferase signal, detected by *in vivo* imaging (IVIS) with the Xenogen 200 system. After euthanasia, the parasite load was quantified using a *Leishmana*-specific qPCR.

2.2 Parasites

A Brazilian strain of wild-type *L. infantum chagasi* (MHOM/BR/00/1669) was maintained in hamsters by serial intracardiac injection of amastigotes. Parasites were grown as promastigotes at 26°C in liquid hemoflagellate-modified minimal essential medium (Berens, R. L., R. Brun, and S. M. Krassner. 1976. A simple monophasic medium for axenic culture of hemoflagellates. J. Parasitol. 62:360–365**.**). Parasite subcultures were grown to stationary phase, and metacyclic promastigotes were enriched on a density gradient as described previously (Yao, C., Y. Chen, B. Sudan, J. E. Donelson, and M. E. Wilson. 2008. *Leishmania chagasi:* homogenous metacyclic promastigotes isolated by buoyant density are highly virulent in a mouse model. Exp. Parasitol. 118:129–133.).

Transgenic parasites were generated by transfection of the wild-type strain with an integrating construct leading to stable mCherry or luciferase expression. Briefly, the gene encoding mCherry or firefly luciferase was cloned into the XmaI site of pIR1SAT, an integrating vector that was kindly provided to us by Stephen M. Beverley of Washington University, St. Louis, MO (Captul et al. 2007). After electroporation (Charmoy et al. 2010) and selection on semisolid medium, correct insertion was verified by South- ern blotting (data not shown). BALB/c mice were anesthetized and inoculated intravenously (i.v.) in the tail vein with 1x10^6 of *L. infantum chagasi* parasites at various doses, using an insulin syringe.

2.3 Assessment of parasite load by *in vivo* imaging.

Relative burdens of luciferase- expressing *L. infantum chagasi* parasites at the inoculation site were determined as previously described (Thalhofer et al. 2010). Briefly, 1 day after inoculation of neutrophil-depleting or control antibody, metacyclic *L. infantum chagasi* promastigotes expressing luciferase were introduced into female BALB/c mice intradermally in the ear. Mice were inoculated with luciferin intraperitoneally and anesthetized with isoflurane prior to imaging.

2.4 Flow cytometry

Single-cell suspensions were suspended in staining buffer (1xphosphate-buffered saline (PBS), 0.5% heat-inactivated fetal bovine serum (HI- FBS), 0.01% sodium azide) at $5x10^5$ cells/sample and kept on ice. Fc receptors were blocked with 10 µl of normal rat serum for 15 min and incubated with fluorochrome-conjugated antibodies. Cells were subsequently washed two times and fixed in 2% paraformaldehyde (PFA). Leukocytes were stained with the following fluorochrome-conjugated monoclonal antibodies (MAb): anti-CD11b-FITIC clone M1/70 eBioscience, anti-CD11c-eFluor clone N418 eBioscience, anti-Ly6C-PerCP-Cy5.5 clone HK1.4 eBioscience, anti-Ly6G-APC clone RB6-8CS eBioscience, anti-MHCII (I-A/E)-PE-Cy.7 clone MS/114.15.2 eBioscience (myeloid panel), anti-CD4-FITIC RM4-5 eBioscicence, anti-CD8a-PE clone 53-6.7 eBioscicence and anti-CD19-PerCP-Cy5.5 clone 6D5 Biolegend (lymphoid panel). Samples were analyzed by flow cytometry (LSRII and FACSDiva; Becton Dickinson, Mountain View, CA). Flow cytometry data were analyzed by FlowJo software (Tree Star, Inc., Ashland, OR). Markers used to identify specific myeloid cell types by flow cytometry are listed in Table 1 and 2. The total cell numbers in livers, spleens or one marrow, were calculated per organ. Infected cell populations were identified by the presence of internalized mCherry+ parasites.

Myeloid Panel **Lymphoid Panel**

Marker	Fluorophore	Marker	Fluorophore
CD11b	FITC	CD4	FITC
LY6G	APC	CD8	PE
LY6C	Percp-Cy5.5	CD19	PE-Cy7
MHCII	PE-Cy7		
CD11c	eFluor® 450		
L. infantum chagasi	mCherry		

Table 1- Immunophenotyping strategy to define cell subpopulations.

Myeloid cells

Cell Type	Abbreviation	Markers
Myeloid cells		CD11b+
Neutrophils	PMN	CD11b+Ly6G^{hi}Ly6C^{int}
Neutrophils-MHCII	PMN-MHCII	CD11b+Ly6G^{hi}Ly6C^{int}MHCII^{hi}CD11c^{low}
Inflammatory Monocytes	infl Mo	CD11b+Ly6G^{low}Ly6C^{int}
Resident Macrophages	res Mac	CD11b+Ly6G^{low}Ly6C^{low}MHCII^{hi}CD11c^{low}
Monocyte derived DC	Mo derived DC	CD11b+Ly6G^{low}Ly6C^{int}MHCII^{hi}CD11c^{hi}

Resident DCs	Res DC	CD11b+Ly6G^{low}Ly6C^{low}MHCII^{hi}CD11c^{hi}
Neutrophil-DC hybrid	PMN-DC Hybrid	CD11b+Ly6G^{hi}Ly6C^{low}MHCII^{hi}CD11c^{hi}

Table 2- Definition of myeloid population by Flow Cytometry.

Lymphoid cells

Cell Type	Markers
CD4 T cells	CD4+CD8-
CD8 T cells	CD8+CD4-
B cells	CD4-CD8-CD19+

Table 3- Definition of lymphoid population by Flow Cytometry.

2.5 Tissue processing

Mice were euthanized, and ears and the draining lymph nodes were removed. Ears were processed as previously described (Menon et al. 1998). Briefly, dermal sheets were separated and placed in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 0.2 mg/ml Liberase CI (Roche Diag- nostic Systems), 100 U/ml penicillin, and 100 μg/ml streptomycin for 2 h at 37°C. Tissues were processed into single-cell suspensions by gentle agitation with frosted microscope slides and placed on ice. Cells were passed through a 70-μm-pore-size nylon mesh and then processed for flow cytometry or DNA isolation.

2.6 Assessment of parasite load by real-time PCR

Tissue parasite burden in draining lymph nodes was quantified using real-time PCR of total DNA ex- tracted from lymph nodes. Genomic DNA was isolated from mouse tissue using a Gentra Puregene DNA purification system (Qiagen) according to the manu- facturer's instructions. Primers and probes for quantitative PCR (qPCR) were used to amplify the mouse tumor necrosis factor alpha (TNF-α) gene as a positive control and *Leishmania* DNA polymerase kinetoplastid DNA to quantify parasites. qPCR primers and probes were previously described (Weirather et al. 2011). TaqMan probes were purchased from Applied

Biosystems Inc. (ABI), and primers were purchased from Integrated DNA Technologies (IDT). Triplicate measurements of samples from six mice were performed. Data were analyzed using a standard curve generated from *L. infantum chagasi* promastigotes (Livak et al. 2001).

2.7 Cytokine analysis

To measure IFN-γ in the spleen and granuloma cultures (1×10^6 cells, each one), organs were collected and single cell preparations were performed at week 4 or 8 postinfection and stimulated with 10^3 promastigotes of *L. chagasi*-mCherry, in triplicate, on 96-well, round-bottomed plates (Nunc) for 48 h at 37 °C, 5 % CO2. The IFN-γ levels were determined in the culture supernatants by commercial kits (catalog no. 555138 BD OptEIATM Pharmingen), according to manufacturer's instructions.

2.8 Bone marrow derived macrophage isolation

Mice were sacrificed in a high CO_2 environment and had their femurs dissected with muscles and tendons removed while retaining the condyles intact. After that, the bones were harvested and had the muscle excess trimmed. The bones were put in mortar along with about 1 ml of RPMI with 10% FBS. The bones were crushed with the pestle until bone fragments were largely obliterated. The contents were poured into 50 ml and filtered through a nylon net to remove any remaining bone pieces, tissue, letting the cells pass through the net. The cells were centrifuged two times at 1400 RMP for 10 min at 4^{o}C and resuspended in RPMI with 10% FBS containing 20% of L929 cells supernatant producing M-CSF to differentiate mouse bone marrow cells to macrophages. BMDMs were plated in L929 cells media on day 0 and again on day 3. After day 6, the BMDMs are already differentiated and were grown without M-CSF.

2.9 *in vitro* infection of bone marrow derived macrophage

The BMDM cells after 6 days of culture were plated $5x10^5$ cells in 24 flat-bottomed tissue plates. After 24 hours, the cultures were infected with isolated promastigotes metacyclics in a proportion of 5:1 (as described before). The infections were kept for 48 hours at 37°C, 5% CO2 and the non-internalized parasites were removed by washing the cultures twice with HBSS-/-. After that, the cells were cultivated in RPMI with 10% FBS with or without stimuli.

2.10 IL-1β release detection from murine BMMac

BMDM, after 48 hours of infection, as described before, were primed with 10ng/mL of LPS (description) for 3 hours. After the priming, the cultures were washed twice with HBSS+/+ and the cells were stimulated with 5mM ATP, for 15 min they had the medium renewed, 100μg/mL for 18 hours. At the end of 18 hours the supernatants were collected and the IL1-β detection was performed by ELISA.

2.11 *In vitro* bone marrow cell differentiation into dendritic cells

BM from femurs was removed as described before and the red blood cells were lysed using 2 ml distilled water for 5-10 seconds and adding 2 ml of 2x PBS and washed twice RPMI 10%. Cells were adjusted to 1 x 10^6 cells/ml with RPMI 10% FBS, and added rm-GM-CSF (50 ng/ml final concentration) and IL-4 (20ng/mL). The cells were seeded into a 10 cm Petri dish in a volume of 10 ml per plate and cultivated for 6 days in 37°C, 5% CO2 incubator. Each two days half of the medium were collected, with floating cells, centrifuged at 1400 RPM for 10 min at 4ºC, and resuspended in fresh medium containing GM-CSF (50ng/mL) and IL-4 (20ng/mL). The fresh medium with the floating cells was put again into the plates renewing part of the medium. Add 10 ml of this suspension to a sterile, microbiological quality, 10 cm Petri dish, and culture in a CO2 incubator (37°C, 5% CO2). The morphology of the bone marrow derived DCs were analyzed subjecting samples to centrifugation in a Cytospin and stained with DiffQuick (Dade Behring) using light microscopy (magnification, x100).

2.12 In vitro DC infection with *L. infatun chagasi*

For *in vitro* infections of DC, promastigotes of *L. infantum chagasi* (MHOM/BR/00/1669)-mCherry were prepared as described previously. Isolated parasites were opsonized with 5% A/J mice serum and 95% HBSS-/- at 26ºC for 30 min and washed before use. After 6 days of culture, bone marrow derived DC (obtained as described before) (2 x 10^5 cells/ml) were infected for 18 h with opsonized promastigotes at a parasite/cell ratio of 5:1, as described previously (Woelbing et al. 2006). Cells were then washed three times to remove adherent, extracellular parasites. Infection rates of DC and the number of intracellular parasites were determined on samples subjected to centrifugation in a Cytospin and stained with DiffQuick (Dade Behring) using light microscopy (magnification, x100).

2.13 *In vitro* DC migration

Uninfected and infected BMDCs, obtained as described before, were counted and adjusted to $2x10^7$ cells/mL. Using ChemoTx® System with a disposable 96-well cell migration system and 8μm pore size, the bottom plate was set up with cell standard curve in triplicates from $4x10^5$ to $1.5625x10^3$ in 1:1 dilutions in 30ul directly in the wells. After that, the chemoattractants were added raging from 10 to 300 ng/mL for all chemokines used (CXCL2, CCL19, CCL21 e CXCL12) in the HBSS-/- in a final volume of 30μL. Also blanks and only HBSS-/- were seeded into the bottom wells to calculate the random migration. The filter lid were fit on the bottom plate and checked for proper seal and medium contact with the pore. $4x10^5$ DCs in 20ul were added to the top of all but standard wells and the covered plates were incubated for 3 hours in a 37°C, 5% CO_2 incubator. To calculate the number of migrated cells, Calcein AM (Sigma 17783-1MG) was added to achieve a final concentration of 10 μM (0.3ul/well in 30ul) and incubated at 37°C, 5% CO_2 for ~30 minutes. Measure of the fluorescence was performed using fluorimeter (Fluostar Omega) plate reader using an excitation filter of 485 nm (20 nm band width) and an emission filter of 535 nm (25 nm band width). The number of migrated cells was calculated based on the fluorescence of standard curves which they were made independently for uninfected and infected cells.

2.14 Neutrophil isolation from murine bone marrow

Mice were sacrificed in a high CO_2 environment and had their femurs dissected with muscles and tendons removed while retaining the condyles intact. After that, the bones were harvested and had the muscle excess trimed. Bones were flushed using ice cold HBSS-/- with 22g or 25g needle and 10 ml syringe. The cells were transferred into 50 mL conical and centrifuged at 1600 RPM for 10 min at 4 ^oC and resuspended in 2 mL HBSS-/-. The percoll layers were added using glass Pasteur pipette underlying the cell layer adding a Percoll solution in HBSS-/- solution of 52%, 69% and 72%, respectively. The layers were centrifuged at 1450 RPM with no break for 30 min at room temperature. The 69%-78% interfaces were retrieved, diluted in HBSS-/- and centrifuged at 1400 RPM for 5 min and finally resuspended in HBSS-/-.

2.15 *In vitro* PMN migration

After PMN isolation, as described before, cells were counted and adjusted to $1x10^7$ cells/mL. Using ChemoTx® System with a disposable 96-well cell migration system and 3μm pore size, the bottom plate was set up with cell standard curve in triplicates from $2x10^5$ to $1.5625x10^3$ in 1:1 dilutions in 30ul directly in the wells. After that, the chemoattractants were added ranging from 12.5 to 50% in the HBSS-/- for LCM (*Lic* conditioned medium) and from 0.005 to 0.5% for activated ZAS each concentration in a final volume of 30μL. Also blanks and only HBSS-/- were seeded into the bottom wells to calculate the random migration. The filter lid was fit on the bottom plate and checked for proper seal and medium contact with the pore. $2x10^5$ PMNs in 20ul were added to the top of all but standard wells and the covered plates were incubated for 2 hours at 37°C, 5% CO2 incubator. To calculate the number of migrated cells, Calcein AM (Sigma 17783-1MG) was added to achieve a final concentration of 10 μM (0.3ul/well in 30ul) and incubated at 37°C, 5% CO2 for ~30 minutes. Measure of the fluorescence was performed using a fluorimeter (Fluostar Omega) plate reader using an excitation filter of 485 nm (20 nm band width) and an emission filter of 535 nm (25 nm band width). The number of migrated cells was calculated based on the fluorescence of the standard curve.

2.16 Statistical analysis

Statistical significance was assessed using two-way analysis of variance (ANOVA) or a Student's *t* test in Prism (GraphPad) software.

3 – RESULTS

NLRP12 is not required for IL-1β secretion in response to TLR agonist including LPS + ATP stimulation

Given the critical role of other NLR proteins in IL-1β processing, we tested the ability of NLRP12-/- cells to produce IL-1β post stimulation with different stimuli. No significant difference in IL-1β production was detected in NLRP12-/- versus wild-type (WT) BMDM

stimulated with LPS, LPS + ATP, or LPS + Alum (Fig. 1). As a negative control, ASC-/- cells failed to produce IL-1β when properly stimulated (Fig. 1). These results are coherent with the findings in Arthur et al. 2012, when the study showed no differences between NLRP12-/- and WT BM cells and dendritic cells in the production of IL-1β, IL-12p40, IL-6, and TNF-a.

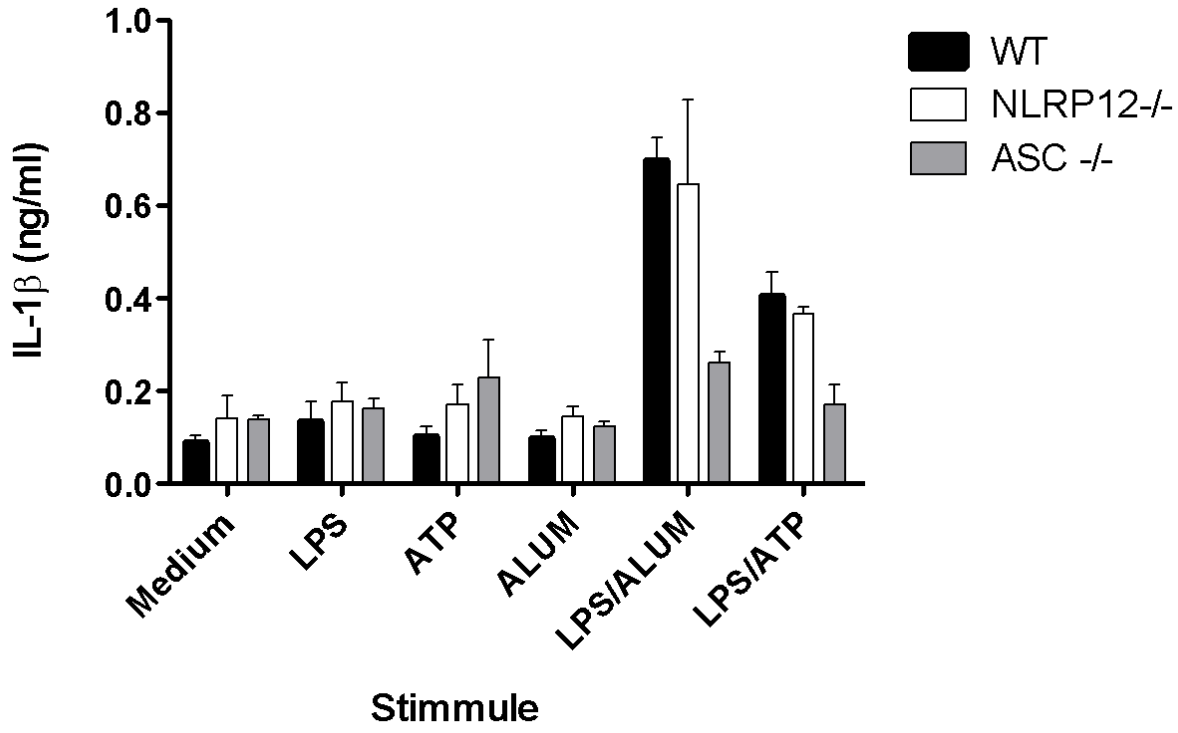

Figure 1- IL-1β released by BMM from WT or NLRP12-/- mice. 5x10^5 murine BMMs, in 500 µl RPMI 10% FBS, were incubated in 24 well plates in indicated conditions and each condition was done in triplicate. Cells were primed with LPS (10 ng/ml) for 3 hours at 37 ºC, 5% CO2. ATP was added to some wells at 5 mM in RPMI 10% FBS, and after an additional 15 min at 37 ºC, 5% CO2 the medium was removed and replaced with RPMI 10% FBS. Alum was used as other stimmuli, where cells were incubated in RPMI 10% FBS with Alum at 100 µg/ml. All cells were maintained at 37 ºC, 5% CO2 for 16 hrs, after which supernatants were collected.

NLRP12 plays a protective role in *Leishmania infantum chagasi* infection

To determine the role of NLRP12 in *Leishmania infantum chagasi* infections, mouse infections were initiated with lines of *Lic* co-expressing luciferase and mCherry. The experiments shown

were initiated with 10^6 metacyclic *Lic* delivered i.v. The progress of infection was followed in individual mice by serial *in vivo* imaging (IVIS). Although mice were shaved and treated with “Nair " to remove fur, the black skin color of C57BL/6 mice still partially quenched light emissions from visceral organs. Despite this limitation, NLRP12 seemed to play a protective role, since observed parasite loads were higher according to the different methodologies in NLRP12-/- compared to wild type mice. NLRP12-/- mice demonstrated an increased infection level in the livers, starting around the week 4 postinfection (Fig. 2B). These results were confirmed by qPCR, which showed that parasite cellular equivalents detected by qPCR of kDNA abundance were higher in the NLRP12-/- mice than WT at week 4, and the number kept increasing with the infection (Fig. 3). NLRP12-/- mice also demonstrated a number of parasites in bone marrow sites, with a peak on day 35 (Fig. 2A and 2B). NLRP12-/- mice also demonstrated higher luciferase activity measured ex vivo after harvesting infected tissues. This higher parasite load was detected by IVIS on day 35, with a peak at day 30 detected by qPCR (Fig. 2A, 2B and 3). As is the case with most mice L. i. chagasi infections the parasite loads peaked in the liver early, but parasite growth lagged in mouse spleens. Thus, we did not detect differences between KO and WT mouse parasite loads by IVIS at these earlytimes. However, the images did suggest a difference in NLRP12-/- infected mouse spleens imaged at later times of infection (Fig. 2A and 2B). The parasite load in the spleen detected by qPCR was the same until week 4, after which the infection in NLRP12-/- mice self resolved although WT mouse infections kept progressing (Fig. 3). Intriguingly, the parasite load kept increasing in the liver although parasites did not proliferate well in the spleens of NLRP12-/- mice, different from the kinetics in WT mice (peak in the liver at week 4). These results suggest a different kinetic of the parasite that is related with the presence of NLRP12, with a possible defect in killing within the liver environment.

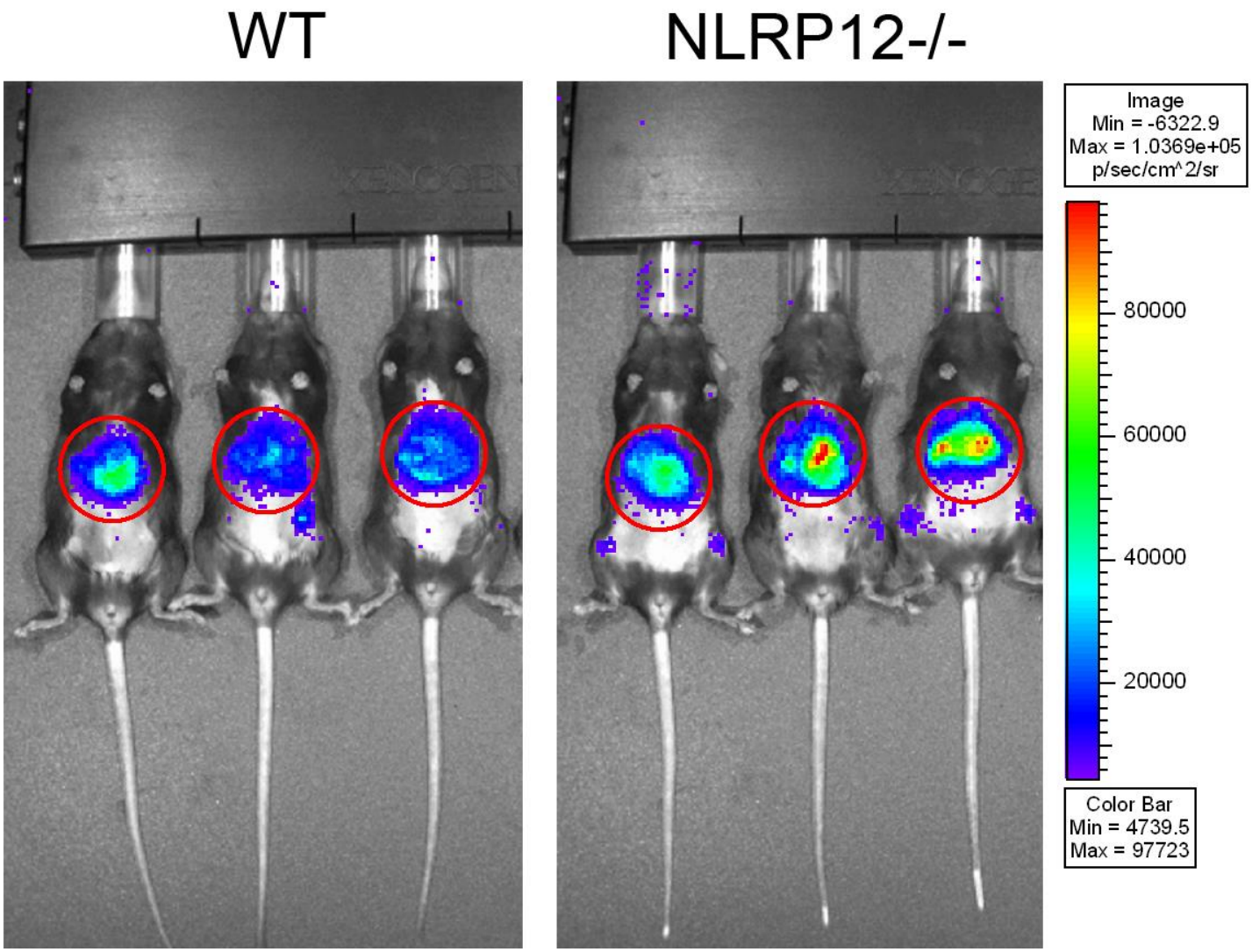

Figure 2- A: *In vivo* imaging analysis by IVIS. WT or NLRP12-/- mice on a C57BL/6 background were inoculated i.v. with 10^6 metacyclic Lic –Luc-mCherry promastigotes through a tail vein. Mice were imaged weekly starting the day of inoculation. Hair was removed by shaving and Nair treatment. Mice were anesthetized with isoflurane, injected i.p. with luciferin (50mg/kg) and imaged after 10 minutes. Data were analyzed using Xenogen IVIS software. In the example, image taken on day 35.

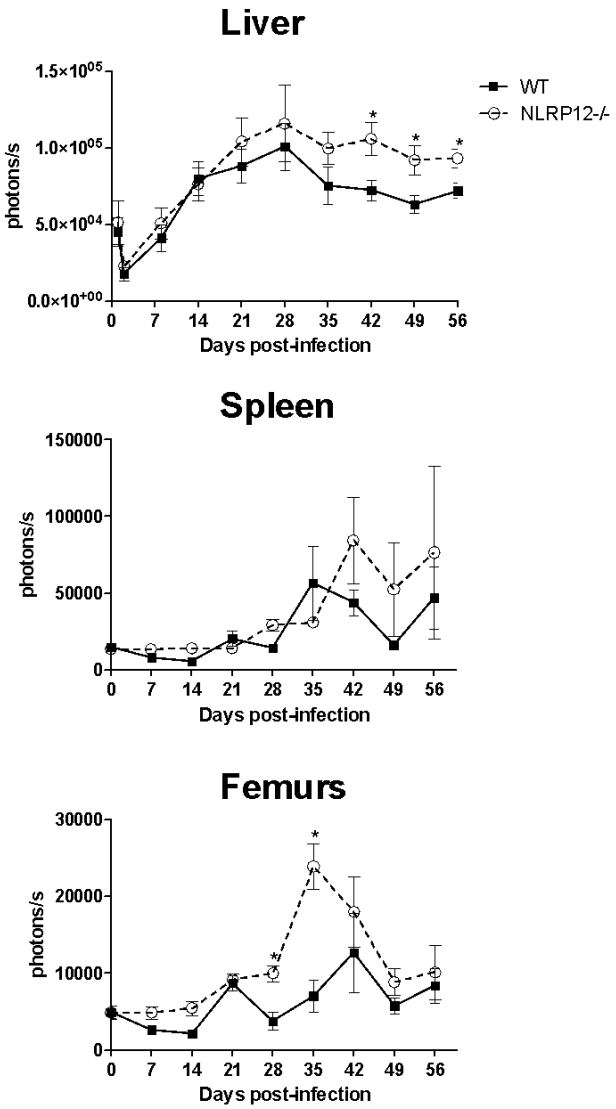

Figure 2 B- Luciferase signal was detected by *in vivo* imaging. ROI were drawn around the regions of the liver, spleen or the femurs and photons/sec were collected from ROI in IVIS images. Data are the result of two experiments with 6 mice/experiment, imaged on sequential weeks. Data are representative of 4 replicate IVIS experiments.

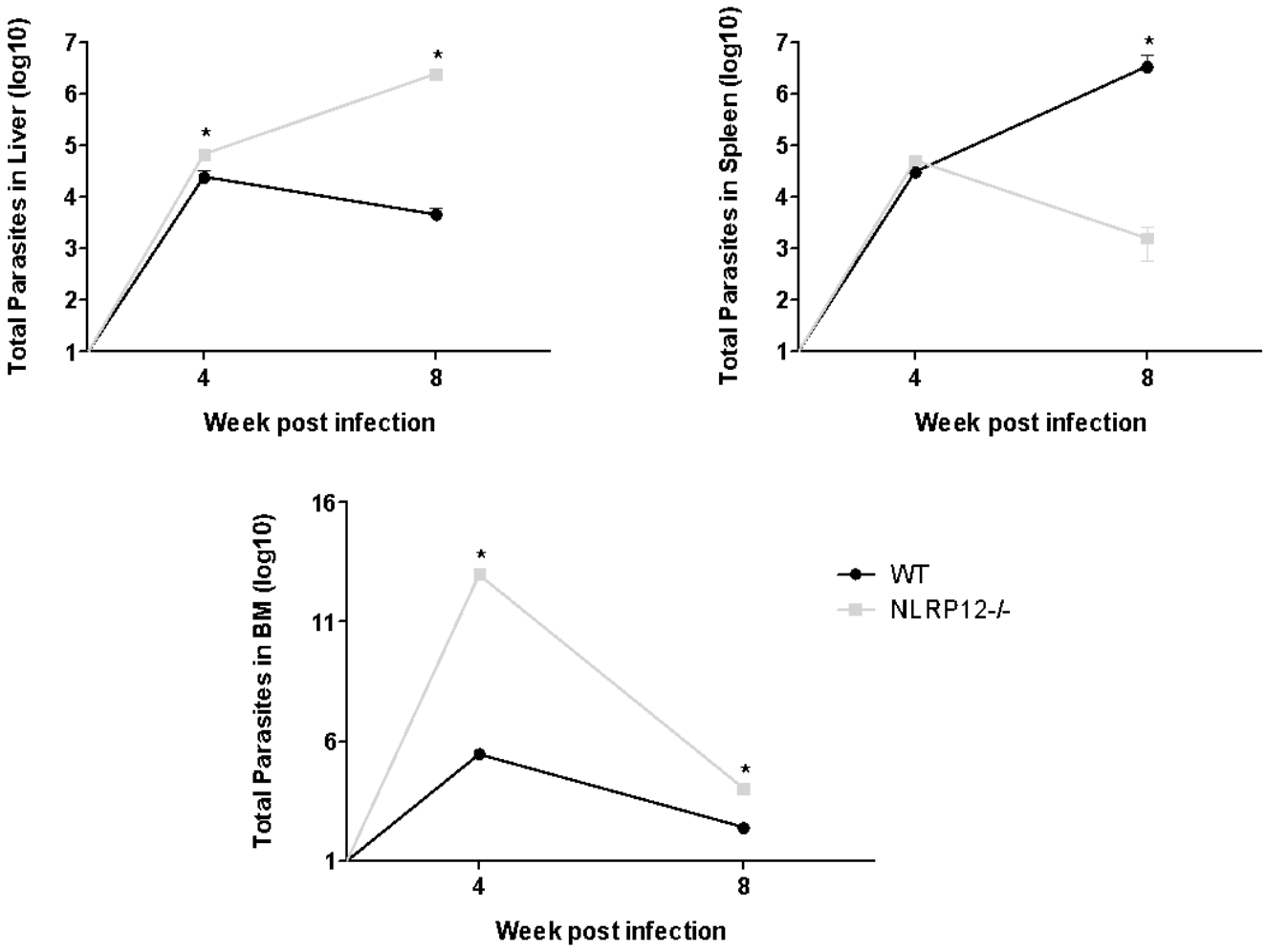

Figure 3- qPCR kDNA detection in the tissues from liver, spleen and BM at week 4 and 8 post *L.i.c* infection. Parasite loads were quantified in the same mice using a quantitative real time PCR assay. DNA was extracted from Livers, spleens or both femurs derived from infected mice, and homogenized in HBSS. Total DNA was extracted from 10% of the homogenate using DNeasy Quiagen. Parasites were quantified by qPCR using a kDNA primer as descried in Weirather et al. 2011. Numbers of parasites were normalized by a standard curve of total DNA from promastigotes mixed with the same organ lysate. Data are the result of two replicate experiments, with 6 mice per time point.

NLRP12 acts as negative regulator of inflammation in *Leishmania infatum chagasi* infection.

To analyze the influence of NLRP12 on the inflammatory process and leukocyte recruitment during *L.i.c* infection, infected mice in C57BL/6 background were infected inoculating 10^6 luc-

mCherry-*Lic* or buffer i.v. and the mice were euthanized at week 4 or 8 postinfection. The organs were harvested and the cell populations were analyzed by Flow Cytometry. The total cell amount per time point in the liver and spleen were calculated. The antibodies used are listed in Table 1 and the immunophenotyping strategy is in Table 2 for myeloid panel and Table 3 for lymphoid panel. The gating strategy for the myeloid panel is demonstrated in Fig. 4A.

The total myeloid cells, represented as CD11b+ cells, were higher during almost the whole infection in the livers of NLRP12-/- infected than WT mice. However, in the spleen the abundance of myeloid cells were the same until weeks 4 postinfection, after which there were fewer numbers of total myeloid cells in this organ. This can be related with the parasite load, which in the NLRP12-/- infected mice was higher and proliferating in the livers of KO mice while parasites decreased in numbers between 4 and 8 weeks infection in WT infected mice. Numbers of parasites in the spleen were higher in KO than WT mice, peaking after week 4 of infection. These results could be explained if the migration of inflammatory cells differed between NLRP12-/- and WT mice, and that myeloid cell infiltration is necessary to carry parasites to the organs.

The graph in Figure 4 also shows the numbers of CD11b+/LY6Ghi/lLY6Clow cells, a region corresponding to the phenotype of PMNs. PMNs (CD11b+/LY6Ghi/lLY6Clow) expanded to a higher number in NLRP12-/- compared to WT mice at week 4 of infection in the liver. However this population decreased in the spleen, compared with WT mice. These results could suggest that differences in some types of myeloid cell migration (e.g., PMNs) could be related to the parasite localization and as consequence the recruitment of this population. As the infection is concentrated in the liver in the NLRP12-/- infected mice, the PMN population is recruited to this site of infection, and to a lesser extent in the spleen, where the infection is lower. This phenomenon was also observed with different myeloid populations, including a novel PMN-MHCII cell, and inflammatory Mo. All were recruited with similar patterns to visceral organs. Even the proliferation of some resident cells (Resident Macrophages and Resident DCs) followed similar patterns of expansion. As such, we needed to determine which cell type was responsible for the defective influx of parasite-harboring myeloid cells.

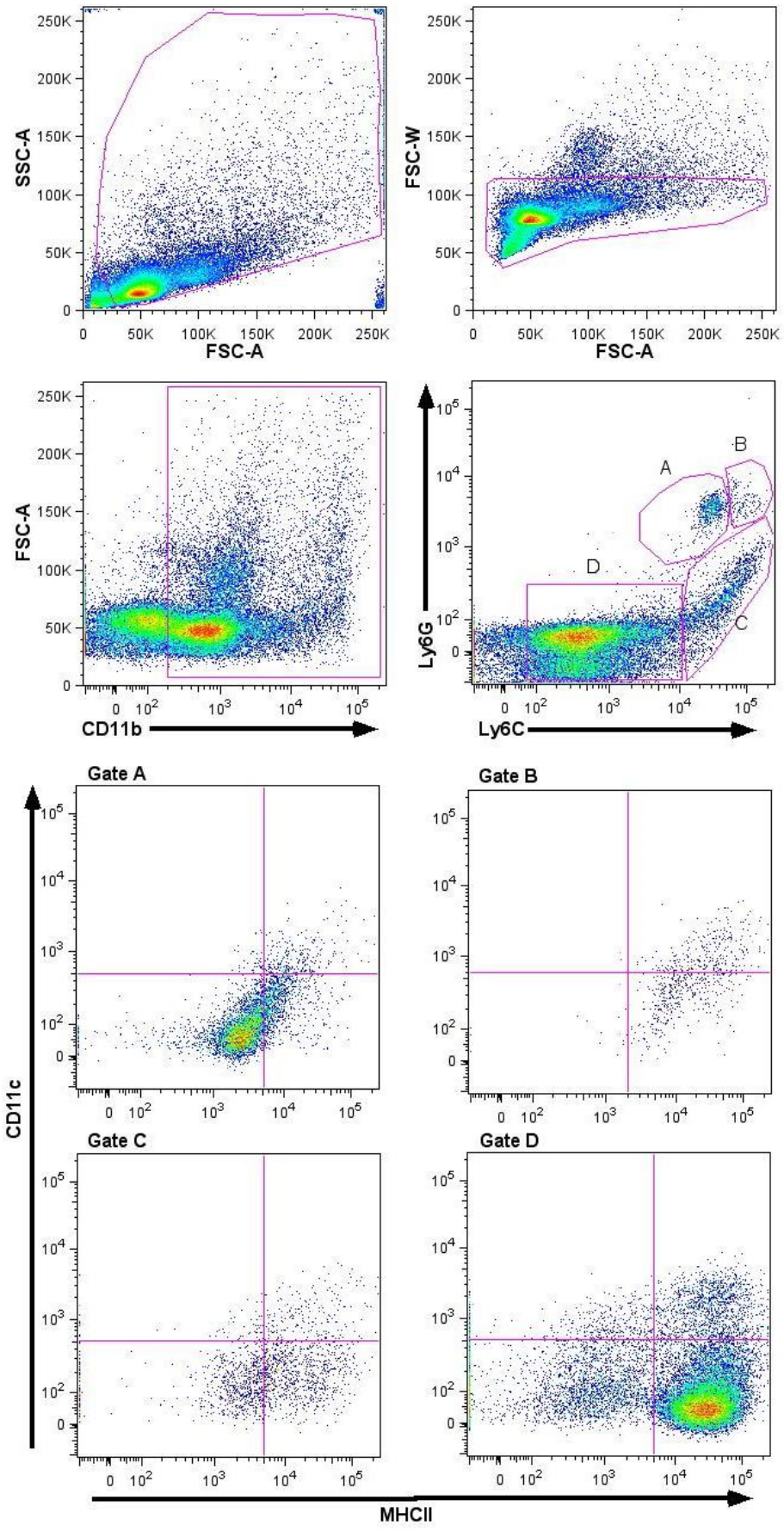
SSC-A
FSC-A
FSC-W
FSC-A
CD11b
Ly6G
Ly6C
A
B
C
D
Gate A
Gate B
Gate C
Gate D
CD11c
MHCII

Figure 4 A.- Gate strategy for myeloid panel analysis. Data were analyzed on FloJo version 8.0 Representative scatter plots of spleen from infected WT C57BL/6 mouse, 4 weeks after infection with 10^6 metacyclic Lic-Luc-mCherry i.v. Gates were set based on unstained samples and compensation controls. Data were derived based upon 50,000 singled events for each sample. $1.5x10^6$ cells per well to stain, all antibodies were diluted in 1:400, 100ul per well was the volume used and all samples were Incubated for 30 min 4 ^oC antibody mixture. Then washed in FACS buffer. Samples, them, were fixed in FACS fixation buffer (2% paraformaldehyde) for 30 minutes 4 ^oC. Washed again 4 ^oC, and maintained in FACS buffer.

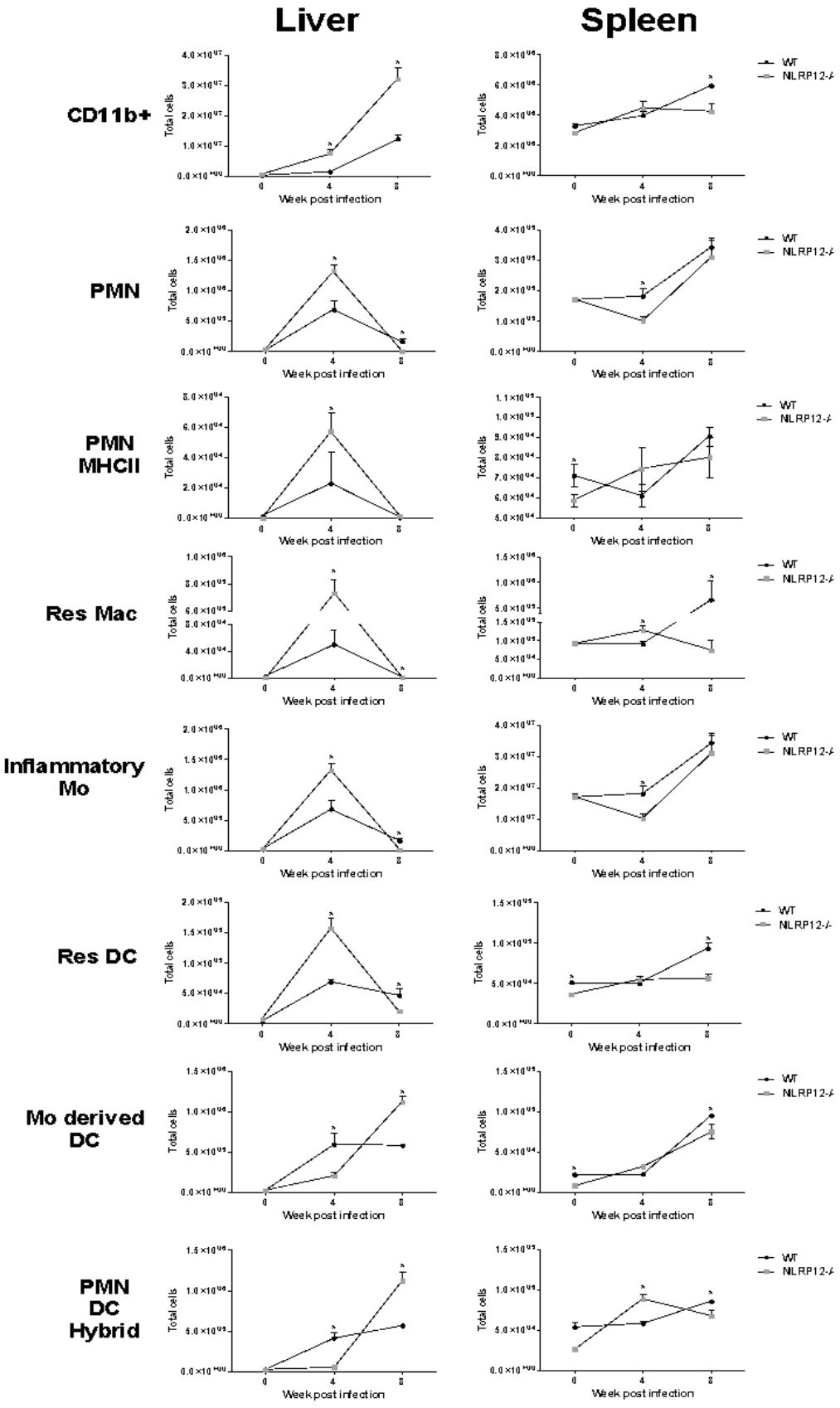

Figure 4 B. Average total myeloid cell populations cells from livers or spleens of mice. Results of 2 experiments with 6 mice each at the indicated time points. Week 0 data were derived from uninfected mice on the day of parasite inoculation. Mean + SD.

However, the derived dendritic cell populations followed a completely different recruitment pattern. Both Mo derived DCs and PMN-DC hybrids showed a delay in migration to the liver in the KO mice (Fig 4B). However these DC populations continually expanded in number in the livers, as at week 8 postinfection and in the meantime these populations showed a reduced number in the NLRP12-/- in the spleen. These results suggest a defect in the migration of these DC subsets from the liver to the spleen at week 8 postinfeciton. These results are corroborative with the findings of Arthur et al. 2010 where the authors showed that DCs from NLRP12-/- mice display a significantly reduced capacity to migrate to draining lymph nodes, in a contact hypersensitivity model. These opposing infiltration patterns of different cell types could implicate one in carrying the parasite to its organ destination (infiltrating DC or MN) and others in clearing the parasites (PMNs).

NLRP12 deficient impaires adaptive immune response in *L.i.c* infection

To determine the role of NLRP12 in the lymphocyte expansion and adaptive response during *L.i.c* infection, C57BL/6 background mice were infected inoculating 10^6 luc-mCherry-*Lic* or buffer i.v. and the mice were euthanized at week 4 or 8 postinfection. The organs were harvested and the cell populations were analyzed by Flow Cytometry, in total cell amount per time point in the liver and spleen. The antibodies used are listed in Table 1 and the immunophenotyping strategy is in Table 3 for the lymphoid panel. The gate strategy for the lymphoid panel was determined as represented in Fig. 5A.

The CD4 population showed a late expansion in the liver of the NLRP12-/- infected mice, comparing with WT (Fig 5B). However in the spleen, this population showed the same behavior in both phenotypes, with minor differences. However in the noninfected mice the presence of NLRP12 seems to regulate the proper proliferation of CD4+ cells. In the NLRP12-/- the CD4+ basal population was increased in the liver, and reduced in the spleen, showing a contrasting proliferating comparing with the WT.

The CD8 population of the NLRP12-/- *Lic* infected mice had a decreased basal level in both liver and spleen, and as the CD4 population, these cells appeared in higher numbers at week 4 of infection in the spleen (Fig 5B). In the liver this population is in a reduced number at week 8 of infection in the NLRP12-/- mice, compared with the WT mice, showing a much lower proliferation than the same population in the WT. Williams et al. 2003 reported that NLRP12

was expressed in human myeloid/monocytic cells and controlled the expression of MHC class I genes in U937 cells and that silencing NLRP12 decreased the expression of MHC class I, so this defect in the CD8+ population can be related at reduced expression of MHC-I in the NLRP12-/- mice.

The CD19+/CD4-/CD8- cells (ploted as CD19+ in the Fig. 5B), considered here as marjority B cells, showed the same expansion patternin both NLRP12-/- and WT phenotypes in the spleen. However, in the liver of the NLRP12-/- infected mice this B cells presented a much higher proliferation, compared with the WT, at the week 4 posinfection. This proliferation of B cell could be related with a stronger TH2 response in the NLRP12-/- infected mice, comparing with the infected WT.

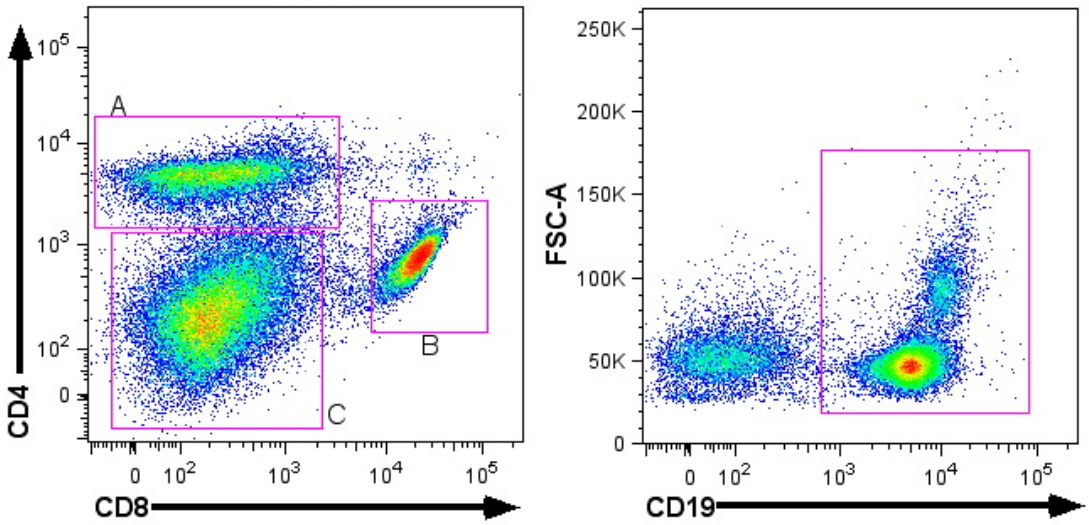

Figure 5 A- Gate strategy for lymphoid panel analysis. Data were analyzed on FloJo version 8.0 Representative scatter plots of spleen from infected WT C57BL/6 mouse, 4 weeks after infection with 10^6 metacyclic Lic-Luc-mCherry i.v. Gates were set based on unstained samples and compensation controls. Data were derived based upon 50,000 single events for each sample. $1.5x10^6$ cells per well to stain, antibodies were diluted in 1:400 for anti-CD4 and CD8 and 1:200 for anti-CD19, 100ul per well was the volume used per sample and all samples were Incubated for 30 min 4 ºC antibody mixture. Then washed in FACS buffer. Samples, then, were fixed in FACS fixation buffer (2% paraformaldehyde) for 30 minutes 4 ºC. Washed again 4 ºC, and maintained in FACS buffer.

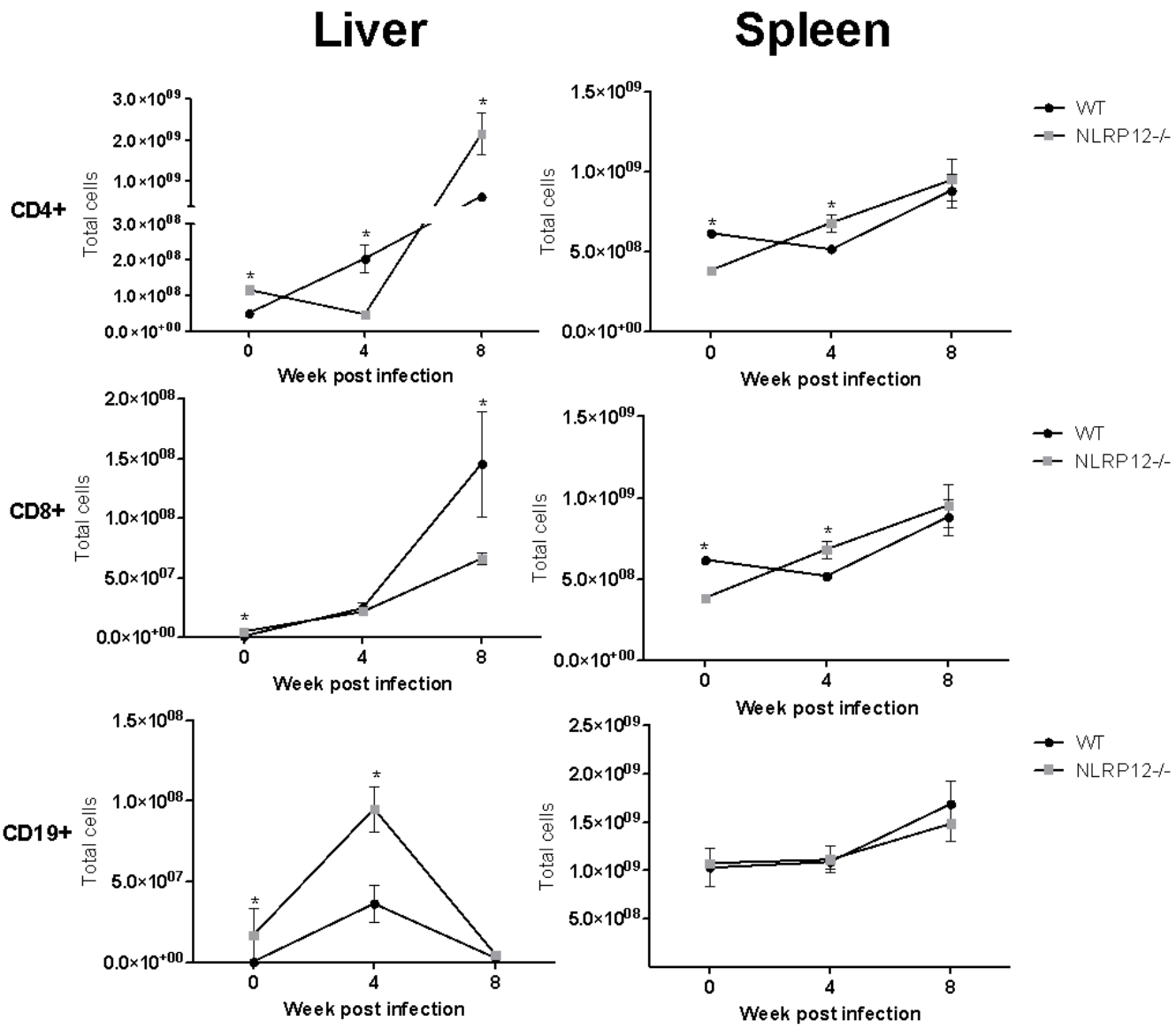
Liver
Spleen
CD4+
CD8+
CD19+
Total cells
Week post infection
WT
NLRP12-/-
0
4
8
*

Figure 5 B- Average total lymphoid cell populations cells from livers or spleens of mice. Results of 2 experiments with 6 mice each at the indicated time points. Week 0 data were derived from uninfected mice on the day of parasite inoculation. Mean + SD. Data are mean +SD.

We assayed the INF-gamma production by ELISA in cultured inflammatory cells from the livers and spleens of mice infected for 4 and 8 weeks with *L. i. chagasi*. The *ex vivo* culture of liver cells from NLRP12-/- at week 4 showed a reduction of the INF-gamma production comparing with the WT when stimulated by the parasites, showing an impairment of this cytokine production in the NLRP12-/- infected mice. At week 8 the culture of cultures cells of both phenotypes showed similar INF-gamma production leves. However the NLRP12-/- infected mice showed an increased CD4+ population at week 4 postinfection, the CD8+ population is decreased, what can explain the reduction of INF-gamma production and the impairment of TH1 response. Also, the CD19+/B cells are much increased in the NLRP12-/- infected mice, which could suggest a TH2 response instead typical TH1 response on the C57BL/6 infected mice background.

INF-gamma production in *ex vivo* cultures of spleens was also measured by ELISA. At week 4 the production of this cytokine was basal in both phenotypes, increasing also in both at week 8 postinfection. However at late infection, week 8, the INF-gamma production from WT cells was much higher than the NLRP12-/- cultured cells.

These results show an impairment of the adaptive response in both infected organs of NLRP12-/- *L.i.c* infected mice. Importantly, the INF-gamma production was reduced. This cytokine is well known to plays an important role in *L.i.c* infection control (Carvalho et al. 2008 and Gazzinelli et al. 2012).

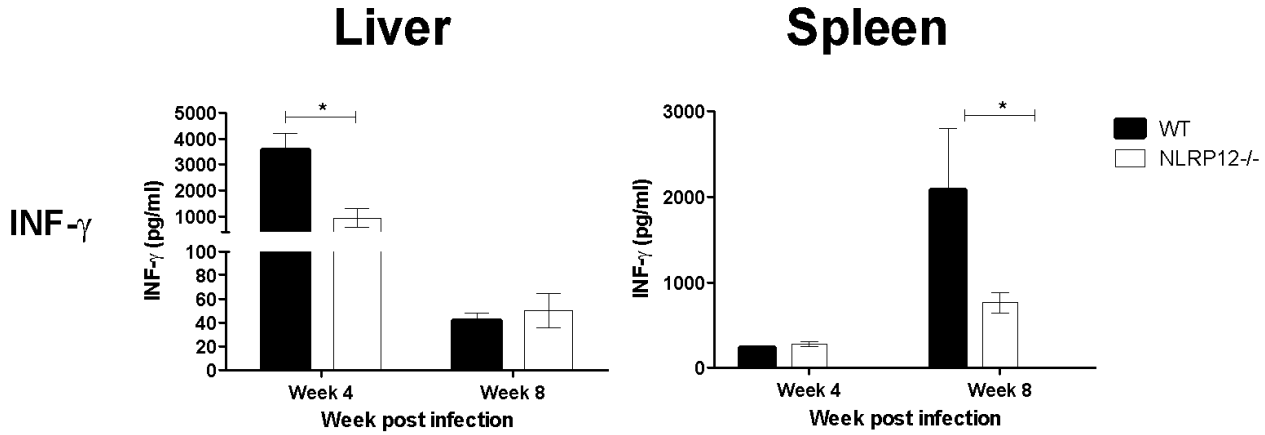

Figure 6- INF-gamma production in cultured inflammatory cells from mice liver and spleen infected at 4 or 8 weeks with *L. i. chagasi*. Cells were cultivated at: $2x10^5$ cells/ per well in 96 well round bottom plates. Incubation was performed for 72 hours and then the cells were stimulated with $6x10^4$ parasites per well (0.3 parasites/cultured murine cell).

NLRP12 regulates DC migration but not neutrophil migration.

To investigate whether the delay in Mo derived DCs and PMN-DC hybrids reaching the liver and the lower amount of these cells in the spleen represents a migration defect, bone marrow of both phenotypes was cultivated in GM-CSF and IL-4 to allow the cell differentiation into DCs subsets and an in vitro migration experiment was performed with these cells. After 6 days in a medium supplemented with GM-CSF (50 ng/mL) and IL-4 (20 ng/mL), both bound and floating cells were collected; the attached cells were harvested with EDTA 5mM. The total collected cells were infected with opsonized stationary phase *L.i.c* for 16 hours. After that, samples of the cultures of both phenotypes, infected and uninfected, were used to prepare slides and had the infection rate analyzed. As illustrated in Fig 7 A, the cultured cells derived from both phenotypes exhibited oval-shaped nuclei and lamellar dendritic processes characteristic of dendritic cells, showing the same pattern on these populations.

The BMDCs from both phenotypes were analyzed for the *L.i.c* infection level, and the DCs derived from bone marrow of NLRP12-/- showed the same level of infection rate as well the same number of parasites per 100 DCs, as demonstrated in Fig. 7 B.

The migration of the BMDCs from NLRP12-/- and WT mice was performed using Chemotx system (NeuroProbe) in a 96 well plate with 8 μm layer filter. Subsets of infected or noninfected cells derived from both NLRP12-/- and WT subsets were tested against different chemokines. All cells were tested through migration to CCR7, CXCR4 and CXCL2 chemokines. Arthur et al. 2010 showed no differences in CCR7 and CXCR4 expression between the NLRP12-/- and WT BMDCs, where CXCR4 is related to immature and mature DC migration and CCR7 to mature DCs. Matsushima et al. 2013 showed that PMN-DC hybrids maintain the CXCR2 expression as well as the neutrophils populations.

With this in mind, BMDCs from NLRP12-/- and WT mice were submitted to in vitro migration experiment towards CXCL12 (CXCR4), CCL19 and CCL21 (CXCR4 and CCR7) as well CXCL1 (CXCR2). The CXCL1 chemokine was used in the experiments in a tempot to evaluate the PMN-DC hybrid population, as they express CCR2 and (Matsushima et al. 2013) we could detect this in the L.i.c infected mice by flow (considered as LY6G+/LY6C-/MHCII+/CD11c+ cells). All chemokines were used and a range from 10 to 300 ng/mL and the migration was allowed for 3 hours in 37 ^{o}C, 5 % CO2 incubator. The migrated cells were detected by Calcein stain by fluorimeter (Fluostar Omega plate reader) and the number of migrated cells were calculated by standard curve of infected or noninfected cells.

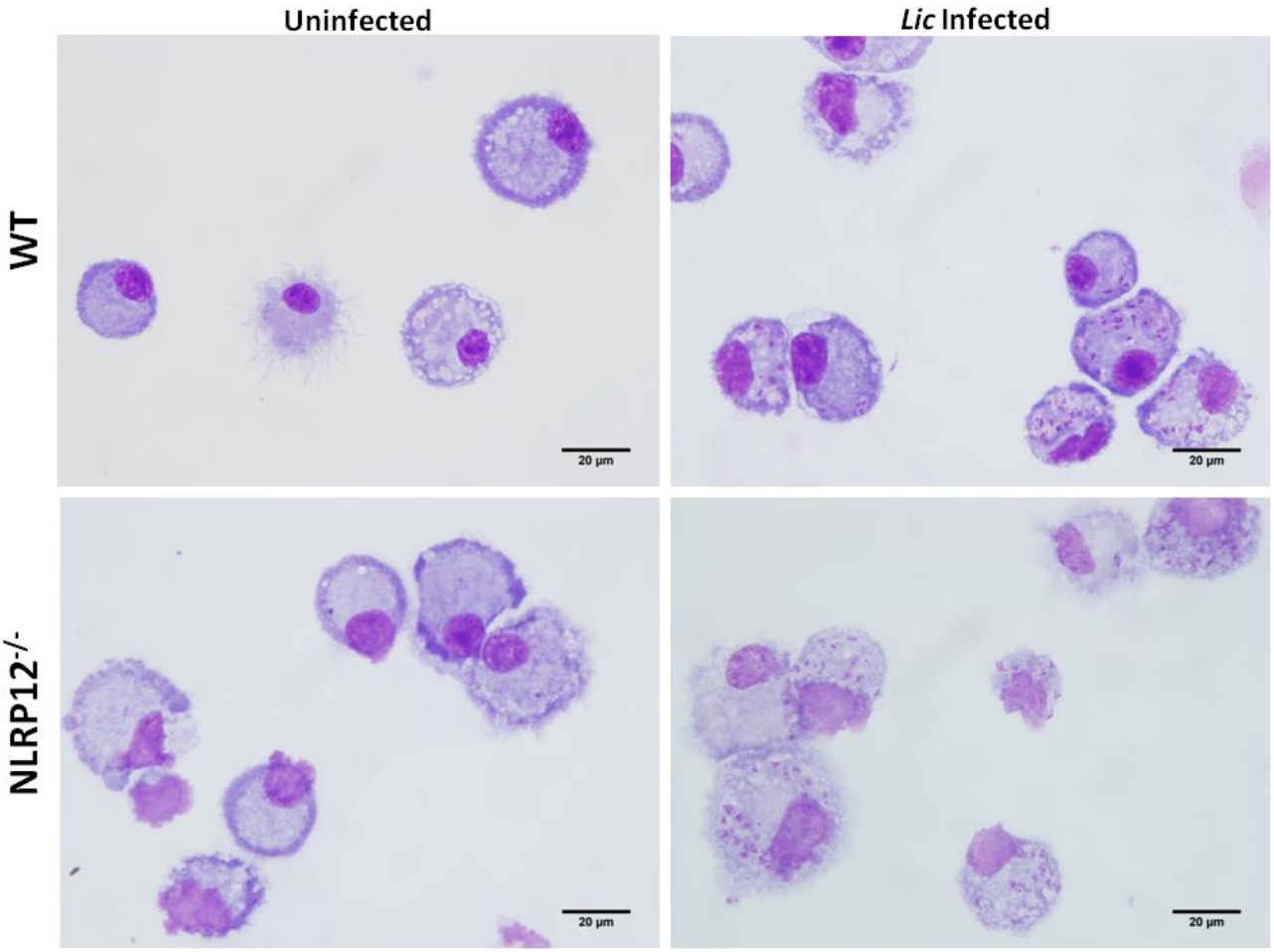

Figure 7 A- Microscopically DC morphology and infection analysis. The slides were stained with Diff Quick (May Grunwald and GIENSA) after they had been prepared by cytospin (basic protocol 100 µl with 1x105 cells, 500 RPM per 5 min). A. Dcs were cultivated in GM-CSF (50ng/mL) and IL-4 (20ng/mL) per 6 days. After that, some were infected with 5:1 opsonized parasites per 18 hours. The culture was washed and slides were fixed in MeOH and stained with Diff-Quik. Shown are representative images at 100x magnification. Bars indicated 20 µm.

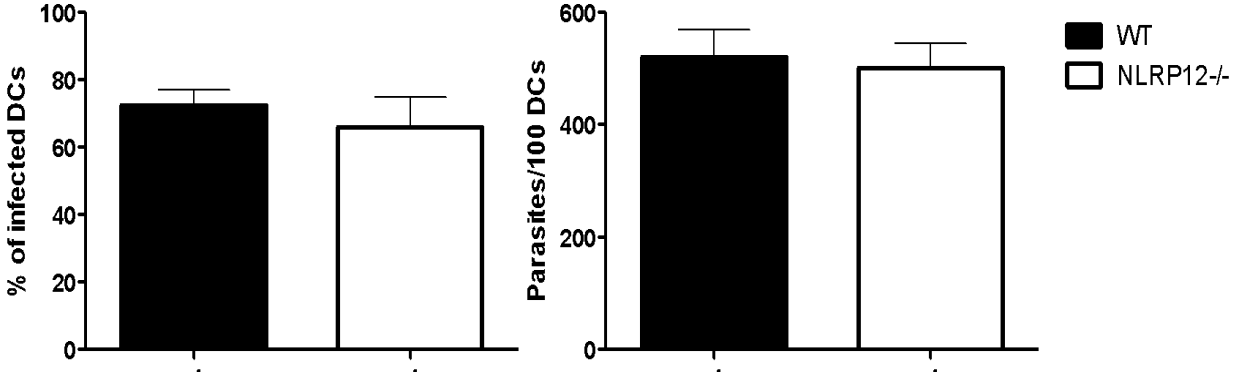

Figure 7 B- Infection rate and parasites number counting of *in vitro* DC infection. Infection was quantified microscopically and the internalized parasites were counted in 2 slides per sample using at least 200 DCs per sample for each condition in two experiments. The slides were stained with Diff Quick (May Grunwald and GIENSA) after they had been prepared by cytospin (basic protocol 100µl with $1x10^5$ cells, 500 RPM per 5 min). DCs prepared and infected as in panel A. Show are the mean +SD

The uninfected BMDCs from WT mice responded to all chemokines tested, showing a dose response curve for the CXCL12, CCL19 and CCL21 (Fig. 8 A). The WT BMDCs also responded to the CXCL1 chemokine, but only with 300 ng/mL of this stimuli. On the other hand, uninfected NRLP12-/- BMDCs did not respond to CXCL12, CCL19 or CXCL1, in any concentration. The CCL21 chemokine was able to induce migration on NRLP12-/- BMDCs at just 300 ng/mL, the highest dose tested (Fig. 8 A). These results confirm the theory that the NLRP12-/- DCs have an intrinsic migration defect and the NLRP12 plays a role in these cells migration.

The infected BMDcs from WT mice had similar migration than the uninfected cells towards CXCL12, CCL19 and CCL21, however this migration was at a lower level, and they could not respond to the CXCL1 stimuli. Also, the *L.i.c* infected NRLP12-/- BMDCs showed a similar migration pattern than the uninfected NRLP12-/- BMDCs, responding just to CCL21 at 300 ng/mL, but as well as the differences between infected and uninfected WT cells, showing that their migration was in a lower level comparing with the NLRP12-/- infected BMDCs (Fig. 8 A).

To explore the differences on the uninfected and *L.i.c* infected BMDCs, a graph with the migrated cell numbers was plotted using just the 300 ng/mL chemokines concentration (Fig. 8

B). In all stimuli tested, the uninfect BMDCs from WT had a higher migration towards the chemokines CXCL12, CCL19 and CCL21, since with the CCL21 the difference between the groups was much more expressive. Even with the NLRP12-/- BMDCs this phenomenon could be detected with the migration against CCL21, the only chemokine that this group responded to, which in the uninfected cells the migration also was in a higher level than the infected cells.
As we detected a lower PMN population in the livers of infected NLRP12-/- mice on week 8 and in spleen at week 4, we performed an *in vitro* migration experiment with neutrophils isolated from bone marrow, to elucidate if this cell amount reduction is due an intrinsic migration defect, or saturation of this cell povoation due a massive PMN infiltration on week 4 in the livers of NLRP12-/- mice. Neutrophils from both phenotypes were isolated from femurs bone marrow by Percoll gradient and had their migration tested using Chemotx system (NeuroProbe) in a 96 well plate with 3 μm layer filter. Different stimuli were used to address the migration behavior of isolated PMN. Activated zymosan (ZAS) were prepared from collected mice blood and used ranging from 0.05 to 0.5 % in HBSS-/- and supernatant from *L.i.c* promastigote culture (LCM) was used in a range from 12.5 to 50% in HBSS-/-. The migration was allowed for 2 hours in 37 ^{o}C, 5 % CO2 incubator. The migrated cells were detected by Calcein stain by fluorimeter (Fluostar Omega plate reader) and the number of migrated cells were calculated by standard curve of PMN cells.
The PMN migration from both NLRP12-/- and WT mice showed a dose response behavior for both stimuli used. Also the amount of migrated cells was not different between theses groups in any stimuli or concentration tested. Also we could not detect any difference in the migration of these groups against CXCL1 (data not shown).

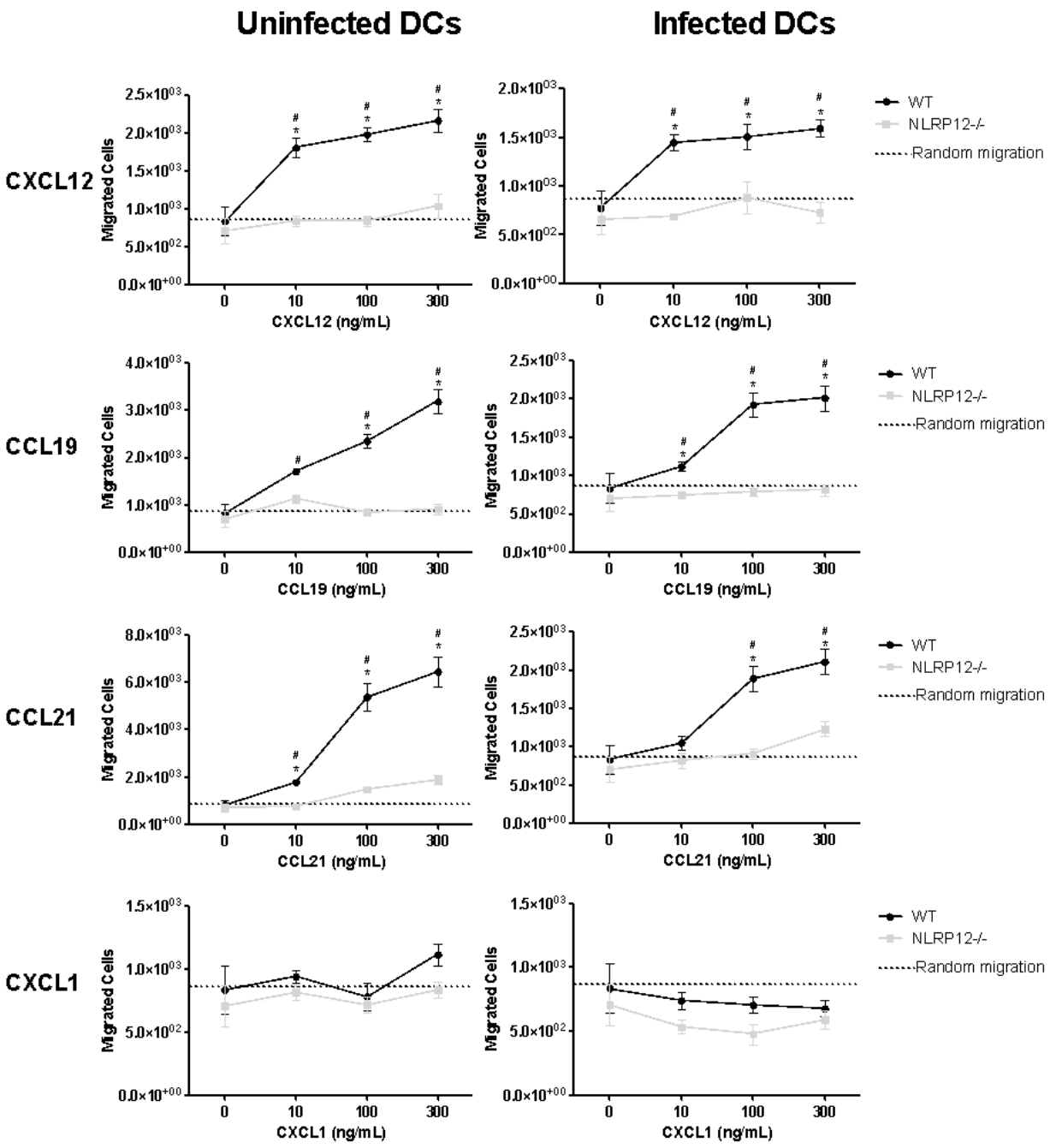

Figure 8 A- Dendritc cell *in vitro* migration. 4x10^5 DCs in 20 µl were incubated in the top chamber of a 8 µm pore size Chemotaxis transwell plate (Peprotech). Bottom wells contained 30 µl of HBSS (-/-) alone or with the indicated chemokines at 10, 100 or 300 ng/ml. Plates were incubated at 37 ºC, 5% CO2 for 3 hours. After removal, cell migrating to the bottom layer were incubated calcein (10 µM – SIGMA) for 30 min at 37 ºC, 5% CO2. Fluorescence was quantified A520-A545 in a plate reader ((Fluostar Omega). Cell numbers were

normalized to a standard curve of uninfected or infected DCs incubated in the bottom well throughout the experiment. Data are the mean +SD of 2 experiments with conditions in triplicate.

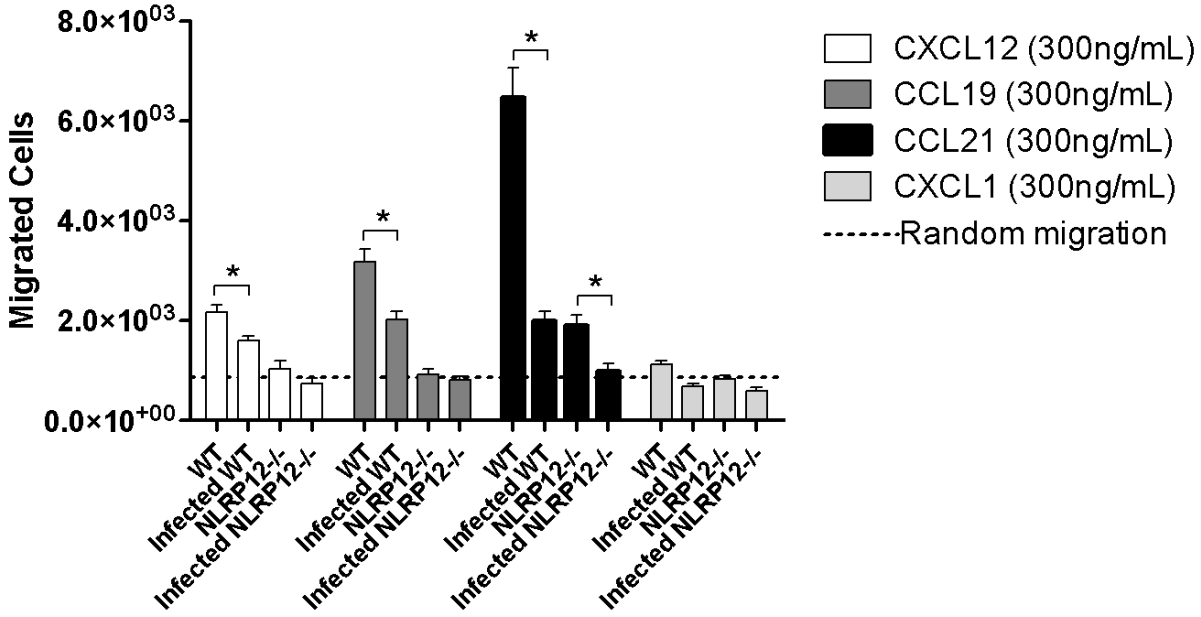

Figure 8 B- Infected vs noninfected DC *in vitro* migration. The same experiments as in Panel C are graphed to emphasize the difference in migration between infected and uninfected DC migration.

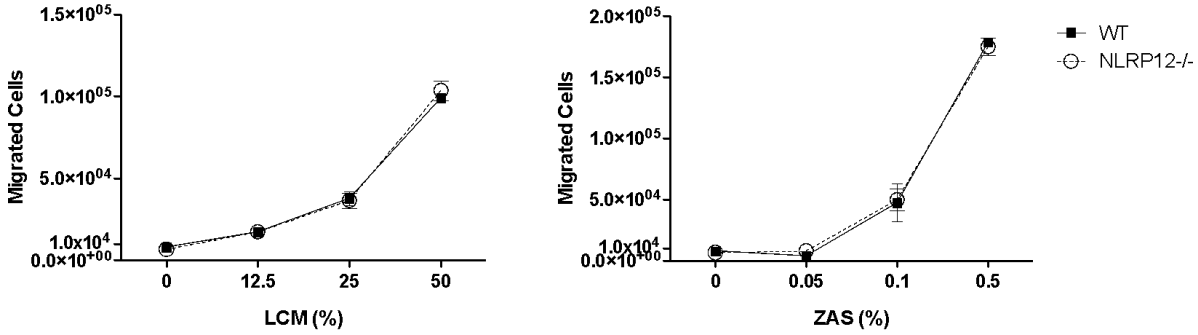

Figure 8 C- Neutrophil *in vitro* migration. 2x10^5 PMNs in 20 µl were incubated in the top chamber of a 3 µm pore size Chemotaxis transwell plate (Peprotech). Bottom wells contained 30 µl of HBSS (-/-) alone or with the indicated percentage of zymosan-activated serum. Plates were incubated at 37 ºC, 5% CO2 for 2 hours. After removal, cell migrating to the bottom layer were incubated calcein (10 µM – SIGMA) for 30 min at 37 ºC, 5% CO2. Fluorescence was quantified A520-A545 in a plate reader (Fluostar Omega). Cell numbers were normalized to a standard curve of PMNs incubated in the bottom well throughout the experiment. Data are the mean +SD of 2 experiments with conditions in triplicate.

NLRP12 regulates the normal leukocyte distribution in the nonlymphoid and lymphoid organs.

We analyzed the basal levels of lymphocyte populations in the livers, spleens and bone marrows of noninfected NLRP12-/- and WT mice at 6-8 weeks old by Flow Cytometry to reveal any differences on these populations that could be related with the NLRP12-/- absent.

We could detect that the CD4+ and CD8+ populations were reduced in the NLRP12-/- mice spleens and bone marrow, however in the livers of these mice, these same populations of CD4+ and CD8+ were increased. These results show an inversion of these lymphocytes. Also, the PMN population was increased in the livers of NLRP12-/- non infected mice, showing an inflammation basal state of this organ in the NLRP12-/- mice.

The basal levels of derived DCs subsets, monocyte derived DCs and PMN-DC hybrids, were found decreased in the spleens of NLRP12-/- noninfected mice, supporting the theory that these cells subsets have an intrinsic migration deffect.

Table 4- Basal levels of cell subsets in tissues of WT or 12 KO mice. Homogenized tissues from uninfected Mice between ages 6 and 8 weeks were stained for flow cytometry as in Figures 4 and 5. Numbers of cells in each category were quantified. Data show the mean +SD of 2 experiments, 6 mice per group. Statistical analyses were done by paired T-test.

LIVER

Myeloid panel	WT	NLRP12-/-
PMN*	2.66E+04 (± 2.40E+03)	3.2E+04(± 1.64E+02)
Res Mac	4.70e+02 (± 8.49E+01)	4.36E+02 (± 1.07E+02)
infl Mo	1.12E+05 (± 7.07E+02)	1.32E+05 (± 1.13E+04)
Mo derived DC	2.22E+04 (± 1.57E+04)	2.23E+04 (± 3.96E+03)
PMN - DC Hybrid	2.71E+04 (± 7.64E+03)	1.76E+04 (± 3.75E+03)

Lymphoid panel	WT	NLRP12-/-
CD4*	5.01E+07 (± 6.94E+06)	1.17E+08 (± 1.48E+07)
CD8*	1.06E+06 (± 3.39E+05)	4.60E+06 (± 7.35E+05)
CD19*	3.40E+05 (± 1.41E+04)	7.44E+05 (± 4.53E+04)

SPLEEN

Myeloid panel	WT	NLRP12-/-
PMN	1.70E+05 (± 8.58E+03)	1.72E+05 (± 1.20E+04)
Res Mac	9.12E+04 (± 2.13E+03)	9.39E+04 (± 4.15E+03)
infl Mo	1.75E+06 (± 8.41E+04)	1.45E+07 (± 9.97E+05)
Mo derived DC*	2.24E+04 (± 1.64E+03)	8.67E+03 (± 2.09E+03)
PMN - DC Hybrid*	5.41E+04 (± 9.96E+03)	2.67E+04 (± 9.87E+03)

Lymphoid panel	WT	NLRP12-/-
CD4*	6.16E+08 (± 1.55E+07)	3.83E+08 (± 4.11E+06)
CD8*	4.94E+08 (± 9.79E+07)	2.57E+08 (± 5.25E+06)
CD19	1.03E+09 (± 2.83E+08)	1.07E+09 (± 2.07E+09)

BM

Myeloid panel	WT	NLRP12-/-
PMN	1.01E+09 (± 2.46E+08)	1.04E+09 (± 4.28E+08)
Res Mac	5.41E+07 (± 8.57E+06)	8.23E+07 (± 1.55E+07)
infl Mo	1.84E+08 (± 6.14E+07)	2.62E+08 (± 1.00E+07)
Mo derived DC	1.78E+07 (± 4.16E+06)	1.02E+07 (± 5.90E+06)
PMN - DC Hybrid	2.14E+07 (± 1.93E+06)	1.45E+07 (± 3.59E+06)

Lymphoid panel	WT	NLRP12-/-
CD4*	4.08E+07 (± 1.06E+06)	1.26E+07 (± 1.66E+07)
CD8*	2.43E+07 (± 9.24E+05)	8.72E+06 (± 1.13E+07)
CD19	3.91E+08 (± 3.06E+06)	3.94E+08 (± 8.00E+06)

4 - DISCUSSION

The *Leishmania* species protozoa cause a spectrum of human forms of leishmaniasis, ranging from spontaneously-healing skin ulcers to fatal visceral disease. Pathologic responses in cutaneous leishmaniasis are associated with strong pro-inflammatory and type 1 cellular immunity. In contrast, progressive visceral leishmaniasis (VL) is characterized by suppression of host immune responses.

The Nod-like receptor (NLR) proteins include more than 20 cytosolic proteins that regulate inflammation and immunity. Activation of some NLRs can cause assembly of an inflammasome leading to IL-1β and IL-18 release. Functions of non-inflammasome forming NLRs, like NLRP6 and NLRP12, are less well understood. NLRP12 (formerly Monarch-1) is a NLR protein that may function as a negative regulator of inflammation, detected by attenuation of the inflammasome-induced NFκB pathway (Wiliams et al 2005). An early study of NLRP12 (Monarch-1/PYPAF7) showed that the protein could influence inflammasomes

(Wang et al. 2002). Consistent with this, NLRP12 has been described to have a pro-inflammatory role during bacterial infection as an important regulator of IL-1β and IL-18 release (Vladimer et al. 2012). In the latter study, NLRP12 deficient mice were unable to control infection with a modified *Yersinia pestis* strain, and had reduced circulating IL-1β and IL-18 and increased spleen bacterial loads (Vladimer et al. 2012). Other studies have suggested NLRP12 as a negative regulator of colonic inflammation and tumorigenesis in a DSS colitis model, and to dendritic cell recruitment (Zaki et al. 2011, Allen et al. 2012, Arthur et al. 2010). It is possible that NLRP6 and NLRP12, and also other NLRs, can play multiple roles in immune function, perhaps dependent upon expression levels in tissues and cells central for specific pathology in various diseases, and cooperation with other signaling molecules.

The role of NLRs and inflammasomes in *Leishmania* infection still needs further investigation. Lima-Junior et al. 2013 showed that the Nlrp3 inflammasome was activated in models of *Leishmania* infection, suggesting its importance for the restriction of parasite replication both in macrophages and *in vivo.* This study addressed responses in inflammasome-deficient mice infected with *Leishmania amazonensis*, *Leishmania braziliensis* or *Leishmania infantum chagasi.* The authors also showed that *Leishmania-induced* NO production through the inflammasome-driven interleukin-1β (IL-1β) production facilitated host resistance to infection, that this resulted from signaling through IL-1 receptor (IL-1R) and MyD88, and that these events were necessary and sufficient to trigger inducible nitric oxide synthase (NOS2)-mediated production of NO. This study addressed the NLRP3 induced inflammasome, but did not explore the roles of non-inflammasoem formation NLRs such as NLRP12.

The role of NLRP12 in infectious diseases has not been extensively studied. And the few studies show that this protein might have a distinct role in different infectious agents. Vladimer et al. 2011 demonstrated that NLRP12 could recognize *Yersina pestis* and increase the IL-1β and IFN-gamma production, revealing a role for NLRP12 in host resistance against this pathogen, Irving et al. 2013 showed no difference in cytokine production, like IL-1β, in NLRP12-/- mice upon LPS stimulation, *Klebsiella pneumonia* infection or *Mycobacterium tuberculosis*. Demonstrating that NLRP12 does not significantly contribute to the *in vivo* host innate immune response to *Klebsiella pneumonia* or *Mycobacterium tuberculosis*. In our models, NLRP12 did not influence the IL-1β production in murine macrophages noninfected or *L.i. chagasi* infected (data not shown), however the *in vivo* experiments showed a role of

the NLRP12 in the host defense, since the absence of NLRP12 caused an increase of the parasite burden in the liver and bone marrow of infected mice. Also we could detect an increased inflammation and leukocyte recruitment in the livers of NRLP12-/- mice, in agreement with the theory that NLRP12 might act as a negative regulator of inflammation (Zaki et al. 2011).

This exacerbated liver inflammation, characterized by the increased amount of PMN, PMN-MHC, and inflammatory monocytes, seemed not to be favorable to healing phenotype, since the parasite load in this organ kept increasing and the IFN-gamma levels were lower. As the *L.i.chagasi* parasite can subvert the host immune response reducing the INF-gamma production and an increasing of the IL-10 and TGF-beta in this infection might be important to suppress the Th1 response production (Gazzinelli et al. 2012), maybe this massive cell infiltration is undergoing a Th2 response avoiding the healing process and facilitating the parasite grow in the tissue.

The increased inflammation released by the NLRP12 absent also caused changes in the kinetic and distribution of parasites with *L.i.c* infection. In the NLRP12 deficient mice the parasites were concentrated or more confined in the liver, where they kept proliferating, and these parasites had a much higher parasite burden peak in the bone marrow, around day 35. In contrast, the parasite burden in the spleen showed no difference by IVIS and a reduction in the NLRP12 deficient mice.

The dendritic cell migration defect, shown in the uninfected and to a higher extent in the infected DCs from NLRP12 deficient mice, might be related with the impairment of the adaptive response in these mice, characterized by the reduction in the IFN-gamma production. Since the cells from NLRP12-/- mice don’t respond to CXCL12, CCL19 and CCL21, the migration to the spleen and antigen present in this organ is compromised, since CCL21 is a chemokine related to lymphatic vessels DC migration and CCL19 to lymphoid tissues, as spleen (Cravens et al. 2002). Also this DC migration defect can explain the reduced total inflammation in the spleen at late infection, as the derived DCs in the spleens are much reduced in the organs of NLRP12-/- mice, reducing this way, the amount of total myeloid cells in the spleens at week 8 postinfection.

The inhibition of DC migration could represent a stratagem for the pathogens to escape the host immune system. *Leishmania* infection can alter the DC migration pattern to the

lymphoid organs; as well some secreted molecules can also modulate this lymphoid attractant. Products secreted by *L. major* promastigotes inhibit the motility of murine splenic DCs in vitro (Jebbari et al. 2002), and *L. major* LPG inhibits migration of murine LCs (Ponte-Sucre et al. 2001).

However, a few studies indicate that although parasites can be detected in mouse lymph nodes a few hours after infection (Mol et al. 1995), none of the DC emigrants from the skin harbors parasites. The authors conclude that DCs could not be the vehicle that ferries the parasites from the skin to the lymph nodes (Baldwin et al. 2001).

Furthermore, *Leishmania* infection can differently modulate chemokine receptor expression on host cells (Panaro et al. 2004) ans absence of CCR2 shifts the *L. major*-resistant phenotype to a susceptible state dominated by Th2 cytokines (Sato et al. 2000).

Little is known about the interaction of Visceral species of *Leishmania* and DC migration. Some of these few studies showed that DCs from mice with chronic *Leishmania donovani* infection fail to migrate from the marginal zone to the periarteriolar region of the spleen. Defective localization was attributable to TGF-Beta, IL-10–mediated inhibition of CCR7 expression in *L. donovani* infected DCs cells, where defective expression of CCR7 on DCs leads to the development of VL (Ato et al. 2002). Also Ato et al. 2006 showed that a loss of dendritic cell migration leads to an impaired resistance to *Leishmania* donovani infection in mice deficient in CCL19 and CCL21, revealing a role of DCs in visceral leishmaniasis.

However, almost none studies relate *L infantum chagasi* infection and DC migration and this interaction is still unknown. In our experiments, infected DCs from both phenotypes, WT or NLRP12-/-, showed a decreased migration towards the CXCL12, CCL19 and CCL21. However it needs to be determined if it is a reduction of these chemokine receptors due *L.i.c* infection, or a physical phenomenon where the infected cells cannot shrink due the internalized parasites and reach the vessels to the proper migration into lymphoid organs.

As the DCs from NLRP12-/- mice did not respond to any chemokine stimuli, as the WT, and Arthur et al. 2010 showed no difference in CCR7 and CXCR4 cell surface expression between WT and NLRP12-/- BMDC from mice by flow cytometry, other pathway may be involved in this migration defect. Some studies have shown the interaction between NLR family members and cytoskeleton. Misawa et al. 2013 showed that microtubules mediated assembly of the NLRP3 inflammasome in macrophages cell lines while Onen 2006 reported

that full-length PYRIN can be colocalized with microtubules and the actin cytoskeleton. Chemokines mediate their effects via interactions with seven-transmembrane-domain glycoprotein receptors coupled to a G protein-signaling pathway (G-protein-coupled receptors or GPCRs). To avoid prolonged activation of the receptors, GPCR complexes are endocytosed and either recycled back to the plasma membrane or sorted into the degradative pathway (Mellado et al. 2001). The mechanism by which the receptor number is regulated on the cell surface, however, is unclear. Receptor number on the cell surface is a balance between the rate of internalization and the rate of replacement (recycling and new synthesis) (Mueller et al. 2002). That can lead us to hypothesize that maybe NLRP12 can have an interaction with the cytoskeleton and or chemokine complexes internalization or even interaction with GPC receptors, playing a role in this chemokine pathway.

For the first time the PMN-DC hybrid population, a novel and not well-understood leukocyte cell population, was detected in *Leishmania* infection. Oehler et al. first described this population in 1998, when the group described molecular changes in neutrophils isolated from human blood and cultivated with GM-CSF, IL-4 and TNF-alpha that include neo-expression of the DC-associated surface molecules as CD1a, CD1b, CD1c, HLA-DR, HLA-DQ, CD80, CD86, CD40, CD54, and CD5, and downregulation of human neutrophil markers as CD15 and CD65s. Later, Iking-Knert et al. in 2001 and 2004 described the same phenomenon that they called “transdifferentiation of polymorphonuclear neutrophils”. In the first study, the group showed that human PMNs cultivated with GM-CSF, INF-gamma, IL-4 and TNF-alpha escaped from apoptosis, and protein synthesis was induced, as MHC-II, CD80 and CD86. Moreover, CD83, thought to be specific for dendritic cells was synthesized, while typical markers of PMN, including CD66b, CD11a/CD11b/CD11c, CD15, and CD18 were preserved. This phenomenon seemed to be forgotten until more recently, when the group of Takashina - Japan, in 2013, again characterized this population, using the term “PMN-DC hybrids” to empathize the idea that the PMN upon GM-CSF could acquire an hybrid phenotype which cells express markers of both neutrophils (Ly6G, CXCR2, and 7/4) and DCs (CD11c, MHC II, CD80, and CD86) (Matsushima et al. 2013). They also exhibit several properties typically reserved for DCs, including dendritic morphology, probing motion, podosome formation, production of IL-12 and other cytokines, and presentation of various forms of foreign protein antigens to naïve CD4 T cells. These cells also retained intrinsic abilities of

neutrophils to capture exogenous material, extrude neutrophil extracellular traps, kill bacteria via cathelicidin production and are MPO positives. Although it was not the focus of this study, we could follow up the PMN transdifferentiation into PMN-DC hybrids during L.i.c infection in both NLRP12 and WT mice. The progression of this differentiation could be elucidated by the MHCII expression, since we could show the formation of PMN expressing MHCII population, and finally a population expressing both MHCII and CD11c markers, that indicates dendritic cell immunophenotyping. However we could detect the presence of these PMN-DC hybrids in *L.i.chagasi* infection, the role of this population in *Leishmania* infection is completely unknown.

The PMN population in the NLRP12 deficient mice did not show an intrinsic migration defect, since they migrated properly to different stimuli, including activated zymosan and medium supplemented with *L.i.c* antigens. They also migrated at the same level of the PMN from WT mice against CXCL1 (data not shown), different from what was shown in Arthur et al. 2010, where they described a reduced migration of these cells to a not specified CXCL1 concentration point.

The inflammatory monocytes and PMN showed the same migration pattern in the in vivo experiments depending on the phenotype. In the case of the WT, these cells first reach the livers around the week 4 postinfection and then they leave this organ and reach the spleens around week 8 postinfection. In the NLRP12 deficient mice, the infl Mo and PMN infiltration has a higher peak at week 4 on infection in the livers, and then these populations reduce in number being replaced by Mo derived DC and PMN-DC hybrids. Also in the spleens, these populations only reach this organ at week 8, which can explain the not well established infection in the organ, since lower cells harboring parasites are migrating to the spleen, as Mos, PMNs, Mo derived DCs and PMN-DC hybrids.

Macrophages are the main host cells for *Leishmania* amastigotes. With this in mind and the fact that in the NLRP12 deficient mice the res Mac population was increased in the livers of these mice during the whole infection, it is possible that this higher amount of host cells could lead to a higher parasite load in this organ, without the proper immune response. Likewise, the res DCs in the liver had the same expansion behavior than the res Macs, however at week 8 the DC population was decreased in the NLRP12-/- mice, while the Macs still increased in these animals. Since the res Macs and Res DCs didn’t increase in numbers during

the infection in the spleens, and Macs are the preferentially host cells for these parasites and together with the DCs they are important antigen presentation cell to lymphocytes in the spleen this lack of expansion of myeloid resident cells in the spleens can be related to the not established infection in the spleens as well as the impairment of adaptive response in the NLRP12 deficient mice.

The NLRP12 showed to have a role in the leucocyte proper organ distribution or populating, since in the NLRP12 deficient noninfected mice these populations showed a different pattern compared with the WT. The spleens of NLRP12 noninfected mice showed a decreased number of CD4+ and CD8+ cells, as the bone marrow also did. But also in the spleen, we could detect a reduction of the derived monocyte and neutrophils derived dendritic cells population, which can be explained by the migration defect of these cells. The liver showed a steady state for inflammation or a pre-inflammed state once the PMN amount in this organ is increased in the NLRP12 mice as well as the CD4+ and CD19+ cells.

The CD8+ cells in the livers of the NLRP12 deficient mice showed a lower proliferation rate, which could be explained by the co-expression of NLRP12 and MHC-I demonstrated by some studies. Williams et al. 2003 reported that NLRP12 was expressed in human myeloid/monocytic cells and controlled the expression of MHC class I genes in U937 cells and that silencing NLRP12 decreased the expression of MHC class I. This apparent correlation between NLRP12 and MHC class I expression has also been highlighted in a human transplantation setting (Hernandez-Ruiz et al. 2011). In this case the lack of proper stimulation of the CD8+ cells could promote a lower proliferation of this cell subset in the main site of infection. That also could help to explain the lower INF-gamma production in the NLRP12 deficient mice.

As the NLRP12 has a role in the gut microbiota homeostasis (Zak et al. 2001 and Irving et al. 2012) and alterations in the microbiome population and/or changes in gut permeability promote microbial translocation into the portal circulation that delivers blood directly to the liver (Son et al. 2010). Liver is the organ most exposed to gut derived toxins (bacteria and bacterial products), due to the portal circulation. Furthermore, non-immune cells including hepatocytes, hepatic stellate cells, endothelial cells, and myofibroblasts also contain the functionally active inflammasome complex (Bawa M. & Saraswat V A 2013). Studies at the mRNA levels support the expression of NLRP1, 2, 3, 6, 10, 12, and NLRC4 in the liver (Yang

et al. 2012). So, maybe NLRP12 has a more tuned role in the interaction between the gut microbiota and the liver inflammation, where in the absence of NLRP12 the microbiota can transpass bacterial content to the portal system, reaching the liver and making the higher leukocyte recruitment, as PMN, CD4+ and CD19+ cells.

Macrophages are the final host cells for multiplication of the intracellular parasite *Leishmania*. However, polymorphonuclear neutrophil granulocytes (PMN), not macrophages, are the first leukocytes that migrate to the site of infection and encounter the parasites (Laskay et al. 2003, Talhofer et al. 2011). The ability to survive and maintain infectivity in PMNs subsequently enables these organisms to establish productive infection is well known upon *L. major* infection. In this infection model the parasites can use granulocytes as Trojan horses before they enter their definitive host cells, the macrophages when taking up apoptotic cells (infected PMNs) silences macrophage killing activity leading to a survival of the pathogens (Laskay et al. 2003). However few studies have addressed the role of neutrophils in visceral *Leishmania* infection (Talhofer et al. 2011). As in the NLRP12 deficient *L.i.c* infected mice they have an increased inflammatory environment in the liver, the previously increased PMN population in this organ before the *L.i.c* infection could favor the settlement of *L.i.c* infection in the livers, acting like the inflammatory model as in *L. major* infection in the skin. However more details on these features needed to be addressed to clarify this theory.

Contrasting with the cutaneous *Leishmania* infection that triggers a strong inflammation, visceral visceral leishmaniasis is characterized by suppression of type 1 immunity and absence of inflammation (Anderson et al. 2007, Kira et al. 2003). Curiously, the increased inflammation on this NLRP12 deficient mice model with visceral leishmaniasis caused by *L.i. chagasi* did not result in a healing phenotype, but an increased susceptibility. Is known that the infection with *L.i.c* can subvert the immune response decreasing the INF-gamma production and increasing the amount of IL-10 and active form TGF-β cytokines (Ansari et al. 2011, Wilson et al. 1998, Wilson et al. 1996). Also these parasites can impair the innate microbicidal activity avoiding the intracellular killing in these cells (Hsiao et al. 2011, David B Sacks, Alan Sher 2002). Moreover, without the proper antigen presentation in the spleen for the lymphocytes by the DCs, this increased inflammation is just recruiting more host cells that are not competent to clear the parasites, leading to a higher susceptibility.

All these features mentioned before, as higher susceptibility to *L.i.c* infection, DC migration impairment, lower CD8+ expansion, decreased INF-gamma production released in *ex vivo* cultures, and a higher lymphocyte B population (CD4-/CD8-/CD19+) taken together, suggest that the NLRP12 deficient mice seemed to swap the immune polarization subtype characteristic to C57BL/6 *L.i.c* infected mice and reveal a protective role of the NLRP12 in *Leishmania infantun chagasi* infection.

5 – CONCLUSION

We investigated the role of NLRP12 in visceralizing leishmaniasis using a murine model with NLRP12-/- (KO) mice and *L. infantum chagasi.* Our study suggests that NLRP12 is essential in the progression of VL.

These data suggest that NLRP12 plays a protective role in VL, associated with the regulation of inflammation, demonstrated as the recruitment of both neutrophil populations (PMN and PMN-MHCII), inflammatory monocytes at the time of peak parasite growth.

Also the NLRP12 has a role in the monocytic and granulocytic DCs migration, where the impairment of this migration led to an impairment of adaptive response, since the proper antigen presentation is reduced in the spleens. This shows a role of NLRP12 in the interaction of innate and adaptive immune response.

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Capítulo 4

CONCLUSÃO

O imageamento *in vivo* se mostrou uma ferramenta extremamente útil para o monitoramento da infecção visceral causada por *L. chagasi*, por meio da qual se pôde observar em tempo real a evolução da infecção em C57BL/6 infectados com tal parasita transfectado com os genes repórteres para luciferase e mCherry. Tal metodologia poderá ser empregada no futuro para a análise da eficácia terapêutica pré-clínica de drogas nos diferentes modelos de leishmaniose.

Nos estudos realizados com a Dra. Mary Wilson durante o aprendizado da técnica IVIS, foi possível revelar o papel negativo na regulação da inflamação pela NLRP12, em que sua ausência desencadeou uma maior infiltração de neutrófilos e monócitos inflamatórios no fígado, como também uma maior expansão de macrófagos e células dendríticas residentes nesse órgão. Além disso, pôde-se confirmar o papel positivo de NLRP12 na migração de células dendríticas (*in vivo* e *in vitro*), em que na deficiência de NLRP12, as DCs se mostraram não-responsivas a diferentes quimiocinas (CXCL12, CCL19 e CCL21), não havendo adequada migração para o baço. Tal fato pode reduzir a apresentação de antígenos, causando prejuízo por haver redução na resposta imune adaptativa, contribuindo para a maior susceptibilidade à infecção causada por *L. chagasi.* Por fim, demonstrou-se que, sem uma resposta imune adequada, um aumento no recrutamento de células do sistema inato ou da inflamação em infecções por *L.i. chagasi* não resulta em aumento da resistência à infecção.

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